MODULATION OF ION TRANSPORT IN INFLAMMATORY BOWEL DISEASES: Regulatory targets

Tesis Doctoral

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Tesis doctoral para aspirer al grado de doctor presentada por

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RESUMEN

1. INTRODUCTION

El transporte de electrolitos, nutrientes y agua que ocurre en la membrana intestinal es consecuencia de los procesos absortivos y secretores de la misma. El agua se mueve siempre por mecanismos pasivos de un lado a otro del epitelio, mientras que los electrolitos responden a procesos activos de las proteínas transportadoras, jugando un papel muy importante en la regulación del transporte de agua. Así, el movimiento de agua viene regulado indirectamente por la propia regulación del transporte electrolítico. Los procesos de tipo secretor están asociados fundamentalmente al Cl^- (*Ilustración 3*), mientras que los de tipo absortivo se asocian al Na^+ (*Ilustración 4*). Ambos se encuentran perfectamente regulados en condiciones fisiológicas. Existen una serie de factores endógenos y exógenos que contribuyen a ese control. La regulación endógena se lleva a cabo por una serie de procesos paracrinos, endocrinos, inmunológicos y neurocrinos. Las toxinas bacterianas constituyen los factores exógenos más importantes que contribuyen a la regulación. Ambos, convergen en la señalización de segundos mensajeros a nivel del enterocito, como es el caso del AMPc o del calcio.

Por otro lado, la inflamación intestinal está asociada a cambios profundos de tipo fisiopatológico, entre los cuales se encuentran las alteraciones en el transporte iónico epitelial y en la función de barrera del epitelio. Ambos factores contribuyen decisivamente al desencadenamiento de la diarrea, y la disrupción de la barrera epitelial puede además contribuir a la propia reacción inflamatoria [1-3]. Los trastornos del transporte hidroelectrolítico constituyen en general la raíz de la mayor parte de los procesos de tipo diarreico (de cualquier etiología), de forma que las alteraciones de la motilidad desempeñan típicamente un papel secundario o accesorio. En general, la diarrea puede asociarse tanto a procesos agudos con mayor o menor componente inflamatorio (intoxicaciones alimentarias, diarreas infecciosas, colitis pseudomembranosa, etc.) y de etiología conocida, como a la inflamación crónica, particularmente la Enfermedad Inflamatoria intestinal –EII– (colitis ulcerosa y enfermedad de Crohn) [4].

Dicha EII se caracteriza, pues, por una inflamación crónica y recurrente del tracto digestivo, de etiología y patogénesis aun desconocidas, pese a los grandes avances que se han realizado en la identificación de los mecanismos fisiopatológicos. Se alternan periodos de exacerbación de los síntomas, seguidos de intervalos más o menos prolongados de remisión de los mismos [4, 5]. Hoy en día dichos síntomas carecen de un

tratamiento efectivo que cure la enfermedad de forma definitiva, por lo que éste está destinado únicamente a mejorar la sintomatología (*Ilustración 3*, *foto 2*, *tabla 1* y *2*).

Pese a que no se conocen los mecanismos responsables de la iniciación y mantenimiento en el tiempo del proceso inflamatorio intestinal, se considera que en su fisiopatología están implicados componentes de tipo genético, ambiental e inmunológico.

Desde el punto de vista mecanístico la diarrea puede estar producida por un incremento en la secreción, por una disminución de la absorción, por una fuga de líquido propiciada por defectos en la barrera epitelial, o por una combinación de estos factores. Sin embargo, el escenario con el que nos encontramos en la EII, con diarrea de tipo crónico, es diferente. Así, podemos identificar alteraciones a nivel de secreción y a nivel de absorción intestinal.

- **Alteraciones en la secreción**

Numerosos mediadores proinflamatorios se comportan como agentes secretagogos *in vitro*, como las prostaglandinas [6], los leucotrienos [7, 8], el factor activador de plaquetas [9], la histamina [10], la serotonina [11], la IL-1β [12, 13], el TNF [14, 15], las especies reactivas de oxígeno y nitrógeno [16], e incluso homogenados de tejido humano afectado por EII [17]. Sin embargo, tanto el transporte iónico basal como la respuesta secretora del tejido inflamado están invariablemente inhibidos, tanto en humanos como en modelos animales de características diversas [17-23]. Por tanto, la secreción iónica no es responsable de la diarrea y en cambio se inhibe en la inflamación crónica, lo que puede interpretarse como una adaptación protectora ante la cronificación del proceso diarreico. La comprensión de este mecanismo tiene un evidente interés, tanto en lo que respecta a la EII como a las posibles implicaciones fisiopatológicas y farmacológicas en la diarrea en general. Además, la inhibición del transporte se prolonga más allá de la resolución de la inflamación [17-24] y afecta también a zonas distales al foco inflamatorio [25].

Investigaciones llevadas a cabo por nuestro grupo de investigación han puesto de manifiesto que el defecto en la secreción iónica está relacionado con una producción reducida de AMPc en el epitelio, lo que reduce la activación del canal CFTR (*Cystic Fibrosis Transmembrane conductance Regulator*) y en consecuencia la secreción basal y estimulada [26]. Aunque se desconoce la causa de la disminución de los niveles de AMPc, existe una clara implicación de la submucosa, dado que la disección de esta capa anula, al

menos parcialmente, la inhibición de la secreción [26, 27]. Otros autores han puesto de manifiesto que el óxido nítrico (NO) puede estar implicado, debido a que la inhibición cede parcialmente con el uso de bloqueantes de la iNOS como el L-NIL (L-N6-(1-iminoetil)lisina) [18, 28]. El NO ejerce acciones antisecretoras indirectas (operando sobre leucocitos) y directas (inhibiendo la adenilato ciclasa –AC- epitelial) [29, 30]. Sin embargo, la influencia del NO en este contexto es en la actualidad controvertida, dado que la secreción inducida por estimulación colinérgica no se ve afectada y la inhibición de iNOS no se asocia siempre a la resolución de la respuesta inhibida. Además el NO ha sido implicado en la diarrea inflamatoria por otros autores [31, 32], y es un agente secretagogo *in vitro* [33] que ha sido propuesto como agente mediador de la respuesta a laxantes [34, 35]. Por tanto, existen diversos factores propuestos como reguladores del sistema de secreción epitelial cuya contribución efectiva ha de ser clarificada. Las citoquinas proinflamatorias (IFNγ, TNFα) tienen un efecto prosecretor agudo pero reducen la expresión de transportadores como la ATPasa Na^+/K^+, el NKCC1 y tal vez el CFTR, aunque los resultados son contradictorios [21, 36, 37]. En general, no se han descrito otros mecanismos de actuación para las citoquinas, ni está claro hasta qué punto el efecto sobre la expresión de transportadores afecta a la secreción, particularmente si tenemos en cuenta que la inhibición es rápidamente reversible *in vitro* [26]. Un resumen de dichas alteraciones puede verse en la *Ilustración 14*.

- **Alteraciones en la absorción**

La absorción de Na^+ (y agua) está incuestionablemente inhibida en el intestino inflamado, tanto en humanos como en modelos animales [22, 38, 39] . Desde el punto de vista mecanístico este fenómeno está poco caracterizado, aunque se ha relacionado con alteraciones en la actividad y/o expresión de transportadores, fundamentalmente la ATPasa Na^+/K^+, el canal ENaC y el transportador NHE3 y DRA [40] [41]. Las citoquinas TNFα e IFNγ y el NO pueden estar implicados en estas acciones [38, 42]. A pesar de la importancia clínica de este fenómeno, se desconoce la reversibilidad o no de esta disfunción. También en este caso las citoquinas proinflamatorias parecen jugar un papel importante a nivel de expresión de transportadores, aunque existen datos contradictorios al respecto (*Ilustración 15*).

Todos estos hallazgos de tipo regulador a nivel de absorción y secreción, añadidos a las alteraciones a nivel de expresión de los distintos transportadores iónicos, son la clave para explicar el estado hiposecretor del transporte hidroelectrolítico del enterocito en la EII.

Es de destacar que en estudios previos a la parte experimental de esta tesis doctoral se ha llevado a cabo un examen exhaustivo de la situación actual del transporte iónico transepitelial en el intestino inflamado, dando lugar a la publicación de dos revisiones publicadas en revistas de alcance internacional [43, 44].

2. OBJETIVOS

Los objetivos iniciales de esta tesis doctoral se centraron en el estudio del efecto de distintos alimentos funcionales en las alteraciones del transporte hidroelectrolítico intestinal en la inflamación colónica, así como una mejor caracterización de la fisiopatología de dichas alteraciones en la EII. Los alimentos funcionales a estudiar fueron el glicomacropéptido bovino y distintos oligosacáridos, como fructooligosacáridos, inulina, oligosacáridos de la leche de cabra y AHCC (Active Hexose Correlated Compound). Sin embargo, en contra de nuestras expectativas, estos agentes mostraron una pérdida sustancial de modulación del transporte iónico, pese a su bien establecida actividad antiinflamatoria intestinal en ensayos preclínicos en modelos de EII. Por ello, decidimos desplazar nuestro campo de investigación a una profunda caracterización de las alteraciones del transporte hidroelectrolítico de la EII como tal, ya que aun hoy día, numerosos interrogantes quedan en el aire, pese a los esfuerzos de diversos grupos de investigación a nivel internacional.

Así, proponemos 5 objetivos principales en esta tesis doctoral:

1. Llevar a cabo una profunda caracterización de las bases moleculares que hay detrás del defecto a nivel de transporte hidroelectrolítico en el intestino inflamado. Especialmente se busca estudiar si alteraciones a nivel del transportoma pueden explicar este estado hiposecretor.

2. Estudiar el origen de la relación entre niveles bajos de AMPc y la inhibición del transporte iónico en la EII.

3. Identificar los factores relacionados con la regulación del AMPc epitelial a través de la estimulación de la proteína G_i.

4. Validar nuestros resultados en muestras humanas, así como mediante el uso de técnicas complementarias, en particular a nivel de genómica funcional.

5. Llevar a cabo un ensayo técnico por análisis de Western blot debido a la necesidad del uso de controles de carga aplicado a las proteínas transportadoras entre otras.

3. MATERIAL Y MÉTODOS

Para llevar a cabo estos objetivos, se emplearon diversas técnicas de cultivo y aislamiento celular, estudios de transporte y permeabilidad por cámara de Ussing, RT-qPCR, microarray, Western blot, inmunohistoquímica y determinaciones celulares por ELISA, LDH, silenciamiento celular o determinación intracelular de AMPc, así como un estudio experimental por colitis inducida por TNBS.

4. RESULTADOS

Nuestros resultados experimentales pueden ser divididos en tres grandes bloques:

- **BLOQUE I.** Para llevar a cabo el estudio de las bases moleculares subyacentes a la inhibición del transporte iónico en la inflamación intestinal, realizamos un análisis genético por microarray comparando colonocitos de rata control con colonocitos procedentes de colitis inducida por TNBS. De las secuencias genéticas alteradas por la inflamación, aproximadamente la mitad están aumentadas, y la otra mitad disminuidas respecto al control (*Tabla 9*, F*igura 4*). Después de un análisis detallado de estos cambios, las alteraciones en el transportoma no explican la situación actual de transporte del intestino inflamado (*Ilustración 17*). Sin embargo, las proteínas reguladoras parecen jugar un papel importante a este nivel. Así, puesto que la vía del AMPc en también objeto de nuestro estudio, observamos alteraciones a nivel de encimas responsables de la síntesis y degradación de este segundo mensajero, como son la AC y la fosfodiesterasa. Concretamente, tres isoformas de AC están inhibidas en inflamación en estos estudios genómicos y postgenómicos: AC5, 6 y 9 (*figura 6*).
- **BLOQUE II.** Puesto a que tras estudiar la expresión de los distintos transportadores de membrana no observamos una correlación directa entre ésta y el estado del intestino inflamado, y puesto a que podría haber vinculación entre los niveles de AMPc y el dicho estado hiposecretor, decidimos centrar nuestros estudios en las diferentes vías que modulan el AMPc y en su estado en condiciones de inflamación. Además intentamos indagar en los mecanismos moleculares que

pueden estar implicados en la inhibición de la AC. Así, estudiamos 4 vías celulares en las que el AMPc está implicado:

A. **CITOQUINAS PROINFLAMATORIAS:** Es hoy día reconocido que las diferentes mediadores proinflamatorios, como las citoquinas IL-1β, TNF α o IFNγ se encuentran sobreexpresadas en la EII. Además, dichas citoquinas han sido implicadas en alteraciones a nivel de transportadores iónicos intestinales. Así, en éste bloque de experimentos estudiamos la relación entre las citoquinas proinflamatorias, los bajos niveles de AC y la reducción de AMPc en condiciones inflamatorias. Para ello, comenzamos con un modelo de cultivo celular *in vitro* con células humanas Caco-2. Tras la preincubación 24h con dichas citoquinas, encontramos inhibición de las tres isoformas de AC (5,6 y 9) en los grupos tratados frente al control (*Figura 10*), menor concentración de cAMP intracelular (Figura 11), así como un estado hiposecretor de las mismas medido por cámara de Ussing (*Figura 12*). Estos resultados son validados en organoides procedentes de yeyuno de ratón, en donde volvemos a encontrar inhibición de las distintas isoformas de AC en presencia de citoquinas proinflamatorias (*Figura 17*). Además, el silenciamiento celular mediante partículas lentivirales para AC5 en células T84, o la inhibición de AC en tejido colónico de rata control, presentan un estado hiposecretor en estudios de transporte en cámara de Ussing.

B. **ÓXIDO NÍTRICO:** Estudiamos el papel del óxido nítrico en la inhibición del AMPc en inflamación mediante estudios en cámara de Ussing. Ni secuestrador de óxido nítrico carboxy-PTIO (*Figura 25*), ni la inhibición de la iNOS con L-NIL (*Figura 26*), ni el donador de NO NONOato (*Figura 27*), consiguen implicar al óxido nítrico, al menos de forma única, con la inhibición de la secreción iónica en inflamación.

C. **OPIOIDES ENDÓGENOS:** Debido al mecanismo de acción de los opioides endógenos a través de G_i, la proteína que inhibe AC, decidimos estudiar dichos opioides como tal (*Figura 28*), así como un agonista de receptores δ-opioides: UFP-512 (*Figura 29*). En ningún caso se explica el estado hiposecretor en inflamación.

D. **PROSTAGLANDINAS:** Por último, decidimos estudiar otros mediadores inflamatorios implicados en la EII: las prostaglandinas, debido de nuevo, a su mecanismo de acción a través de Gi o Gs, modulando así la AC y las concentraciones de AMPc. Como era de

esperar, todas tienen efectos prosecretores a nivel basal excepto la prostaglandina D_2 (PGD_2) (*Figura 30*), con una acción mediada fundamentalmente a nivel de submucosa. Por ser la principal prostaglandina antisecretora, la PGD_2, nos centramos en su mecanismo de acción en inflamación. La PGD_2 tiene dos receptores, DP_1 y DP_2. DP_1 actúa a través de G_s en AC, aumentando así las concentraciones de AMPc intracelulares, mientras que DP_2 actúa a través de G_i y reduciendo los niveles de AMPc en el enterocito. El bloqueo de DP_2 con ramatroban, revierte parcialmente el grado hiposecretor en colon distal de ratas colíticas en cámara de Ussing. Cuando intentamos reproducir el efecto de PGD_2 en biopsias de colonoscopias de pacientes sanos, observamos un efecto contrario de PGD_2 (*Figura 42*): es prosecretora en condiciones basales. Sin embargo, se observa una tendencia a recuperar este estado hiposecretor en condiciones de inflamación, estudiando la secreción de biopsias procedentes de pacientes con EII (*Figura 43*). Esta diferente actuación de la PGD_2 en muestras control entre rata y humano se debe a una diferente expresión de los receptores DP_1 y DP_2 (*Figura 44*). En ambas especies, sin embargo observamos un incremento de una de las dos encimas responsables de la síntesis de PGD_2, L-PTGD sintasa (*Figura 41* y *46*). Además, llevamos a cabo un estudio del efecto del ramatoban (inhibidor del receptor DP_2), y HQ-L79 (inhibidor de la sintasa H-PGDS de PGD_2) en la colitis inducida por TNBS en ratas, y pese a la reducción de marcadores inflamatorios como MPO, S100A8, IL-1β, INOS, MCP-1 o TNF, ninguno de los dos tratamientos consiguen revertir el estado hiposecretor de la inflamación.

- **BLOQUE III.** En este último apartado se pretende abordar una cuestión de tipo técnico: la propuesta es validar el empleo del Rojo Ponceau, técnica rápida, reversible y económica, como control de carga en la técnica de Western blot, sustituyendo así al empleo de actina. De dicho estudio se publica un trabajo que hoy día cuenta con un total de 96 citas [45].

5. CONCLUSIONES

1. En el modelo de colitis experimental inducida por TNBS en rata, la expresión epitelial de AC 5,6 y 9 está inhibida. Este efecto puede deberse a citoquinas proinflamatorias (IL-1β, TNFα y IFNγ) y contribuye al

defecto en la secreción iónica. El novedoso sistema de cultivo de organoides constituye un valioso modelo de validación no tumoral para dichos resultados.

2. El aumento de la producción de PGD_2 en la colitis por TNBS en rata activa receptores DP_2, con el subsecuente incremento en la función de la proteína G_i, reducción intracelular de los niveles de AMPc e inhibición de la secreción iónica basal. Ni el óxido nítrico no los opiáceos endógenos parecen jugar un papel sustancial a este nivel.
3. Cambios en el transportoma del enterocito no explican el estado de inhibición iónica en la colitis de rata por TNBS.
4. El efecto de PGD_2 en colon no inflamado de humano es sustancialmente diferente al colon de rata, debido a diferencias de expresión en la transducción receptorial. Sin embargo, PGD_2 puede inhibir la secreción iónica en biopsias de pacientes con IBD de manera similar que lo observado en colitis de rata por TNBS.
5. El eje PG_2/ DP_2 juega un rol patogénico en la colitis de rata por TNBS. La inhibición de la síntesis de PGD_2 o la ocupación del receptor DP_2 no revierte el defecto en secreción iónica.

INTRODUCTION

1. GUT PHYSIOLOGY

1.1. ANATOMY OF THE INTESTINE

The intestinal tract, from the esophagus to the rectum, is essentially a tube, whose wall is composed of four tissue layers arranged in a concentric structure as shown in *Illustration 1*: mucosa, submucosa, muscularis externa and serosa [46, 47].

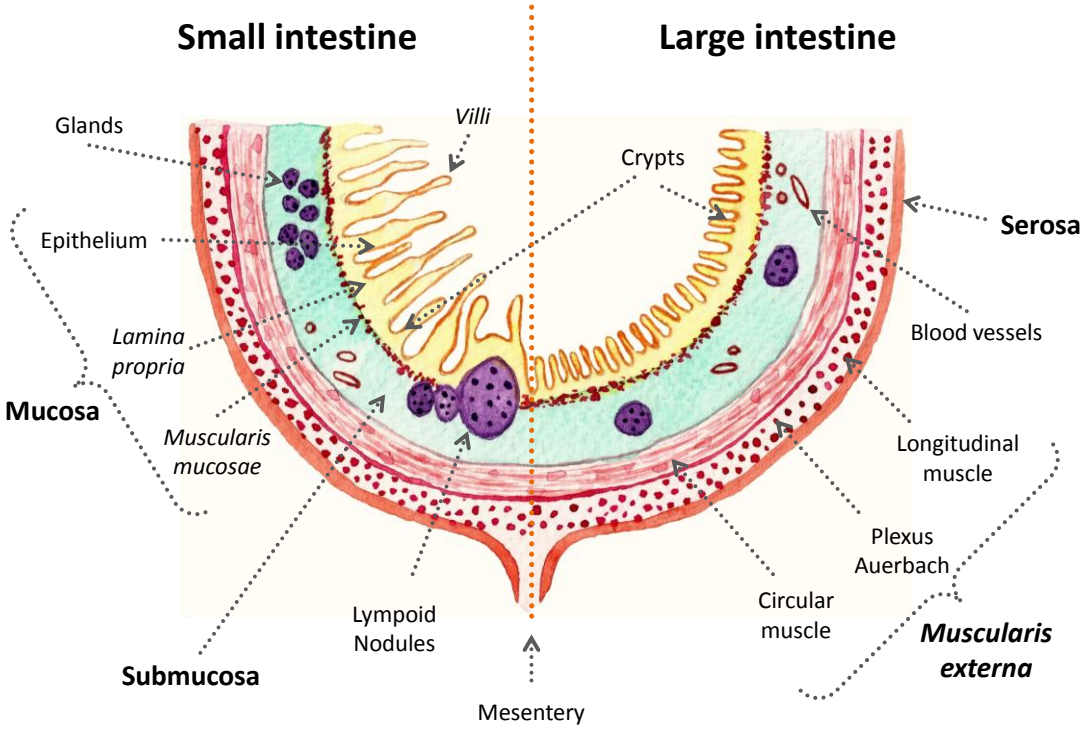


Illustration 1: *schematic representation of the wall structure of the digestive tract.*

1.1.1. Mucosa

The mucosa is, in turn, made up of three layers: an *epithelial lining*, a supporting connective tissue designated as the *lamina propria*, and a thin layer of smooth muscle, the *muscularis mucosae*.

- **Epithelium**: the type of epithelium that is present depends on the functions of the part of the tract that it lines. At some sites it is essentially protective (e.g., the stratified squamous epithelium of the esophagus and anus). At other sites it is secretory (e.g., the mucus-secreting epithelium of the stomach) or absorptive (e.g., the columnar epithelium of the small and large intestines). The epithelium is responsible for the nutrients and water absorption and water secretion, which occur simultaneously, as well as for the compaction of the stool, which depends on water movement across the epithelium. This process is exquisitely regulated to provide adjusted rates of both [46, 47]. Four differentiated cell types mediate the functions of the intestinal epithelium: enterocytes, mucosecreting, enteroendocrine and Paneth cells (*Illustration 2*). The relative abundance of each of these cell types varies markedly within different segments of the intestine.

    - **Enterocytes** are tall columnar epithelial cells, with a basally positioned nucleus. They are polarized cells, with a differentiated apical and basolateral membrane. They contain numerous parallel *microvilli* at their apical surface, forming the striated border visible by light microscopy. A microvillus contains a core of microfilaments comprised of actin, which are anchored to the plasma membrane at the tip. The filaments extend into the terminal web, another network of microfilaments, immediately under the *microvilli*. The terminal web consists mostly of horizontally disposed microfilaments that insert into the zonula adherens of the apical junctional complex. In the intestine the epithelium is not spread out as a flat layer; rather, the intestine has invaginations called crypts and luminal projections called *villi* (excluding the colon). This arrangement augments the epithelial surface tremendously, allowing efficient absorption of nutrients compounds from the intestinal lumen. Strong cell proliferation occurs within the crypt compartment. This process involves stem cells that have long remained elusive, but are believed to reside near the base of the crypt. Cell proliferation is balanced by anoikis (apoptosis induced by detachment from the extracellular matrix) and cell shedding at the other end of the epithelial conveyor belt, (the tip of the villus in the small intestine case, or the top of the colonic crypt in the large intestine; see below). In both the small and large intestine, cells differentiate into four functional cell types as

they migrate: the predominant enterocytes, the mucus-secreting Goblet cells, the peptide hormone secreting enteroendocrine cells and the Paneth cells in the small intestine, which migrate down to the base of the crypt (see below). The tissue therefore has a distinct polarity, with new cells created in the crypt, migrating predominantly upwards into the villus. Here the cells perform their specific functions and are then shed into the lumen 5-7 days from cell division [48, 49]. Crypts can multiply through lateral fission [50]. Enterocytes are joined at their apical poles by tight junctions to neighbouring enterocytes. These junctional complexes include occluding junctions that separate the lumen of the intestine from the lateral intercellular, or paracellular, space, and they are more or less permeable depending on the intestinal segment considered. The impressive functional capacity of the intestine both to secrete and absorb water is largely the result of the extensive functional surface of the intestine, due to the amplification factor brought about by the *villi* and *microvilli*.

- The number of mucosecreting cells, also called **Goblet cells**, increases from proximal (small intestine) to distal (colon and rectum) intestine as the stool becomes increasingly compacted. These cells are glandular simple columnar ephitelial cells whose main function is to secrete mucin, which forms a gel as it is secreted into the apical aqueous space to form mucus. The majority of the cell cytoplasm is occupied by mucinogen granules.

- **Enteroendocrine**, enterochromaffin or Kulchitsky cells represent a small proportion (<1 %) of the cells in the epithelium. They control gut physiology by secreting a variety of hormones including serotonin (they contain about 90 % of the body's store of serotonin), somatostatin, substance P, motilin, vasoactive intestinal peptide and secretin. Multiple subtypes can be defined by the specific intestinal hormones that are produced [51].

- **Paneth cells** reside at the bottom-most positions of the crypts mainly of the small intestine. They are identified microscopically by their localization adjacent to the intestinal stem cells. Most of their cytoplasm is occupied by granules, which consist of several antimicrobial compounds, such as defensins, cryptidins and lysozyme, which are known to be important in immunity and host defense, and play an essential role in the control of the microbial environment of the intestine.

- Finally, some others cell types should be mentioned, such as the **M cells** that cover the lymphoid Peyer's patches [52], **brush/tuft/caveolated cells** [53], and **cup cells** [54].

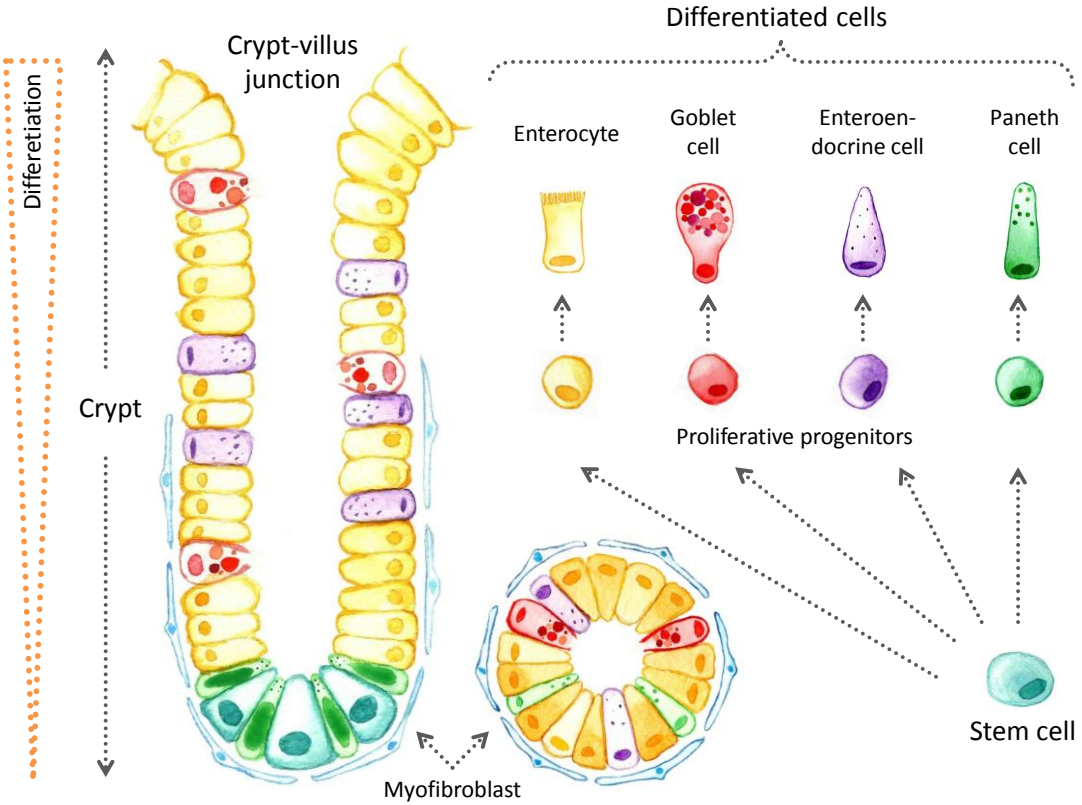


Illustration 2. *Intestinal epithelium cell types.*

- ***Lamina propria* and its associated lymphatic tissue**: the *lamina propria* supports the epithelium and attaches it to the muscularis mucosae. It also contains many lymphocytes and unencapsulated lymphatic nodules, which are collectively referred to as the gut-associated lymphoid tissue (GALT). Also featured are plasma cells, eosinophils, and other migrant cells from the blood. Such lymphoid tissue is a major source of IgA plasma cells, a class of immunoglobulins that can be transported into the gut lumen by the mucosal epithelium. In the small intestine, aggregates of nodules called Peyer's patches are usually located on the

side of the tube opposite to the attachment of the mesentery. In addition, the *lamina propria* brings fenestrated blood capillaries as well as lymphatic capillaries close to the surface epithelium, particularly within projecting *villi* of the small intestine. Hence products of digestion do not have to diffuse very far before gaining access to either type of capillary.

- ***Muscularis mucosae***: this outermost layer of the mucosa consists of two thin layers of smooth muscle fibers that are arranged circularly, in the inner layer, and longitudinally in the outer layer. Its contractile activity permits independent movement and folding of the mucosa, aiding digestion and absorption. Smooth muscle fibers extend from the *muscularis mucosae* of the small intestine to the tip of each villus; their tonus determines the height of the *villi*.

1.1.2. Submucosa

This layer consists of moderately dense, irregular connective tissue. It attaches the mucosa to the *muscularis externa*. It contains larger blood vessels. This layer has a substantial content of elastic fibers and forms the core of mucosal folds. In the duodenum and esophagus, the submucosa also contains mucus-secreting glands. Deep in the submucosa lies a plexus of autonomic nerve fibers and ganglion cells known as the submucosal (Meissner's) plexus.

1.1.3. *Muscularis externa*

The *Muscularis externa* consists of two layers of smooth muscle; the inner one is circular and the outer one longitudinal. The tonus of the inner circular layer determines the overall luminal diameter of the bowel. In addition, the *muscularis externa* undergoes peristaltic contractions that propel the gut contents toward the anus. Like the submucosa, it contains networks of nerve fibers and ganglions cells: these constitute the myenteric plexus of Auerbach, that is situated between the circular and the longitudinal layer of the *muscularis externa*.

1.1.4. Serosa

This outermost layer of the gut wall consists of a single layer of simple squamous epithelial cells, the mesothelium, and a small amount of underlying connective tissue, which is crossed by numerous transverse

blood vessels. In regions of the gut that are attached to the adjacent tissues, the connective tissue is not covered by mesothelial cells, but merges with the connective tissue associated with the surrounding structures. In this case, it is known as an adventitia instead.

1.2. DIFFERENCES BETWEEN SMALL AND LARGE INTESTINE

The intestinal tract can be anatomically divided into two well-defined segments: the small intestine and the large intestine or colon, (*Picture 1: Scanning electron micrographs of mouse intestinal epithelium (taken from Magney et al., 1986)).*

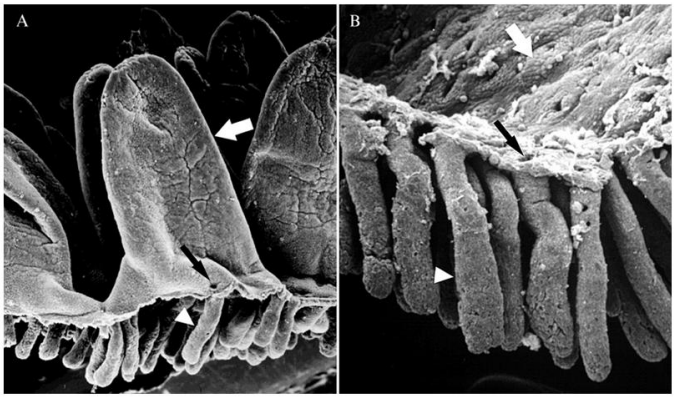
1.2.1. Small intestine

The small intestine is approximately 6-8 meters long, and can be subdivided into three segments: duodenum, jejunum, and ileum. The main functions of the small intestine are to complete the digestion of food from the stomach, to absorb useful products of digestion into blood or lymphatic capillaries, and to produce gastrointestinal hormones. The small intestine contains the four characteristic layers described above. In addition, the mucosa and submucosa are significantly modified at several levels of organization to increase the luminal surface area. These modifications include *plicae circulares*, *villi* and the striated border. Thus, beginning a few centimeters from the pylorus, there are circular or spiral mucosal folds with submucosal cores. These folds are known as *plicae circulares* (valves of Kerckring), and they are most numerous in the proximal region of the small intestine. Another adaptation folding of the mucosa produces *villi*, surrounded by crypts. This organization increases the epithelial surface tremendously. *Villi* are small finger-like projections with a nominal height of 300 to 500 µm. Typically, each *villi* contains a centrally placed lymphatic capillary called a lacteal. The intestinal mucosa contains glands throughout its length. The intestinal glands, or crypts of Lieberkühn, are simple tubular glands that extend through the thickness of the mucous membrane and open into the intestinal lumen at the base of the *villi*. In the more distal part of small intestine, there are the aggregations of nodules called Peyer's patches.

1.2.2. Large intestine

The large intestine consists of the cecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum, and anal canal. The four layers characteristic of the gastrointestinal tract are present throughout. However, neither *plicae circulares* nor *villi* are present. Its surface appears as a flat surface

punctuated by holes corresponding to crypt openings. Goblet cells are more numerous in the large intestine than in the small intestine, and crypts invaginate deep into the submucosa.



Picture 1. *Scanning electron micrographs of mouse intestinal epithelium (taken from Magney et al., 1986).* ***A****: Isolated small intestinal epithelium: white arrow: villus.* ***B****: Isolated colon epithelium: white arrow: flat surface epithelium. In both figures: white arrowhead: crypt; black arrow: orifice of the crypt lumen, visible on the surface.*

2. MOLECULAR BASES: transepithelial ionic transport

In physiological conditions the intestine is exquisitely regulated to provide adjusted rates of both water absorption and secretion, which occur simultaneously. The human intestine secretes about 1000 ml of water daily, along with 1500 ml of pancreatic juice, 500 ml of bile, 2500 ml of gastric secretion and 1500 ml of saliva [55, 56]. This is added to dietary intake, which is highly variable but averages 1500 ml. However, the small intestine readily reduces the amount of liquid entering the colon to 1.5-2 l, where the volume is further reduced by approximately 90% to match the fecal mass quantity, so that the feces are not so dry that they are difficult to expel or so watery that they constitute diarrhea. The result is the daily faecal electrolyte excretion of less than 5 mmol/l Na^+, 2 mmol/l Cl^-, 9 mmol/l K^+ [57] and approx. 5 mEq HCO_3^- [58]. The ileum and proximal or transverse colon constitute a functional unit [59] and absorbs water with moderate efficiency through a low resistance or "leaky" epithelium. Conversely, the distal or left colon is a more efficient stool dehydrating system which operates via a medium resistance epithelium, i.e. with less permeable tight junctions [55]. The mechanism of ionic transport also differs. Osmotic water transport is driven by the vectorial movement of ions from one side of the intestinal epithelium to the other. Thus the expression and regulation of the different intestinal ion transporters are key to the fine tuning of water movement. It is thought that the regulation of epithelial transport lies chiefly in the submucosal plexus and the paracrine influence of fibroblasts, immune cells, etc. In addition, it is widely acknowledged that the central nervous system affects gastrointestinal function.

2.1. Na^+/K^+ ATPase: the driving force

The mechanism of ion transport by the intestinal epithelial cells has been well characterized [55, 60]. The driving force for both ionic absorption and secretion is invariably the electrochemical gradient created by Na^+/K^+ ATPase activity, which is located exclusively at the basolateral membrane of the enterocyte (*Illustration 3*) along the crypt-surface cell axis. This ion transporter is involved in a multitude of cellular functions, including not only the facilitation of membrane transport but also the regulation of cell volume and membrane potential, cell growth, differentiation and cell death [61-63]. The Na^+/K^+ ATPase is composed of two subunits, α and β, of which the α subunit serves transport function while the β subunit is thought to modulate the expression of the pump in the membrane. The Na^+/K^+ ATPase moves 3 Na^+ ions outside the cell in exchange for 2 K^+ which enter the cytoplasm from the basolateral milieu in every cycle.

This energy consuming (active) system produces both an electrical (intracellular voltage negative compared to extracellular) and a chemical imbalance (Na^+ and K^+ are unequally distributed at both sides of the cell membrane). From a thermodynamic point of view, this circumstance favors the entrance of cations (especially Na^+) and the exit of anions and K^+. Because lipid bilayers have an extremely low permeability for ions, channels are required to facilitate their movement across the membrane. Such mode of transport is not coupled directly to energy spending and is therefore passive by definition, although it is ultimately dependent on the activity of the Na^+/K^+ ATPase and thus 'secondarily' active. Since normal cell membrane potential is approximately –70 mV (intracellular side negative), Na^+ ions tend to move inside the cell down the chemical (and electrical) gradient. Similarly, an increase in Na^+ conductance (i.e. an opening of Na^+ channels) will cause depolarization. Na^+/K^+ ATPase requires Mg^{2+} to be active [64].

2.2. Water movement

Fluid movement across gut specialized epithelium is bidirectional, and involves both absorptive and secretory processes. There are two pathways for water transport across the epithelium:

(1) The paracellular route, through the spaces between cell junctions, called the tight junctions (TJ), (*Illustrations 3 and 4*). As a function of cell junction electrical resistance, gastrointestinal epithelia have been classified into: tight (gastric fundus), rather-tight (gastric antrum and colon) and leaky (small intestine) [65]. TJ are relatively leaky in the small intestine, but as mentioned above become increasingly less permeant in the colonic segment. This pathway for water transport has been shown to be limited, especially in leaky epithelium, and is far from being fully understood [66-69].

(2) The transcellular route, which involves the passage of water through apical and the basolateral cell membranes of epithelial cells [66, 68, 70, 71]. This may happen using three different mechanisms: passive diffusion through the phospholipid bilayer [72], cotransport with ions and nutrients [73], and diffusion through water channels called aquaporins (AQPs), (literally, 'water pores') [67, 72, 74-79]. Since the colon mucosa is a tight epithelium characterized by high electrical resistance, it is very likely that water movement takes place mainly through AQPs, (*Illustrations 3 and 4*). The relative roles and contributions of cotransporters and AQPs in the transcellular passage of water are still debated [80]. In particular, it is well accepted that AQPs are responsible for osmotically-driven transmembrane water

movements, while the presence of an active water transport (that could move water against osmotic gradients) is controversial and requires further supporting experiments [73, 81, 82]. The gastrointestinal tract expresses several AQPs, numbered from 1 to 11 [83], but their role is insufficiently investigated [84]. Several AQP isoforms are found in gastrointestinal epithelium, with AQP1, 3, 7, 10 and 11 being the most abundantly expressed in the whole gut. On the other hand, AQP4 and 8 are located exclusively in the stomach and colon, respectively [83]. Caution must be exerted because of the unexpected cellular roles of AQPs not related to water transport, as revealed by AQP knockout mice studies [85-87]. This is the case of AQP1 in cell migration, a function behind processes like angiogenesis, wound healing and tumor growth [85]; or AQP3 in the enterocyte proliferation [86]. [88]

The two pathways are not completely independent, since lateral intercellular spaces may provide a compartment in which both pathways communicate.

2.3. Hydroelectrolytic secretion

Water secretion is linked primarily to chloride vectorial transport from blood to lumen (*Illustration 3. Secretory transporters*). Secretion of electrolytes and associated water is essential for the gut function to moisturize the luminal contents, enabling digestive enzymes to reach their substrates, and maintaining normal stool passage.

Cl^- enters the enterocyte, together with Na^+ and K^+, through the electroneutral **NKCC1** cotransporter ($Na^+/K^+/2Cl^-$), also called SLC (solute carrier) 12A2[1], which is located exclusively at the basolateral membrane [40, 89]. Consistent with the concept of the crypt as the primary site of secretion, NKCC1 exhibits an expression gradient along the crypt-villus axis, and is most abundant at the crypt base {Reynolds, 2007 #199}. The stoichiometry is $Na^+:K^+:2Cl^-$, so that this step is electroneutral. Cl^- can also enter the enterocyte through the basolateral anion exchanger 2 (**AE2**, SLC4A2), which has been identified in the distal colon both in the surface and crypt cells, and exchanges Cl^- and HCO_3^- [90, 91]. K^+ is mostly recycled back to the interstitial space through specific **K^+ channels**. This has the effect of augmenting the cell membrane

[1] **Slc#/SLC#:** HUGO nomenclature for solute carrier. The # symbol indicates gene family; all-capital formatting is specific to human genes *(www.genenames.org/genefamilies/SLC)*.

potential difference, which in turn favors Cl^- exit, which occurs at the apical side via the **CFTR** (Cystic Fibrosis Transmembrane conductance Regulator) channel (ABCC7), strongly expressed in small intestinal and colonic crypts [92].

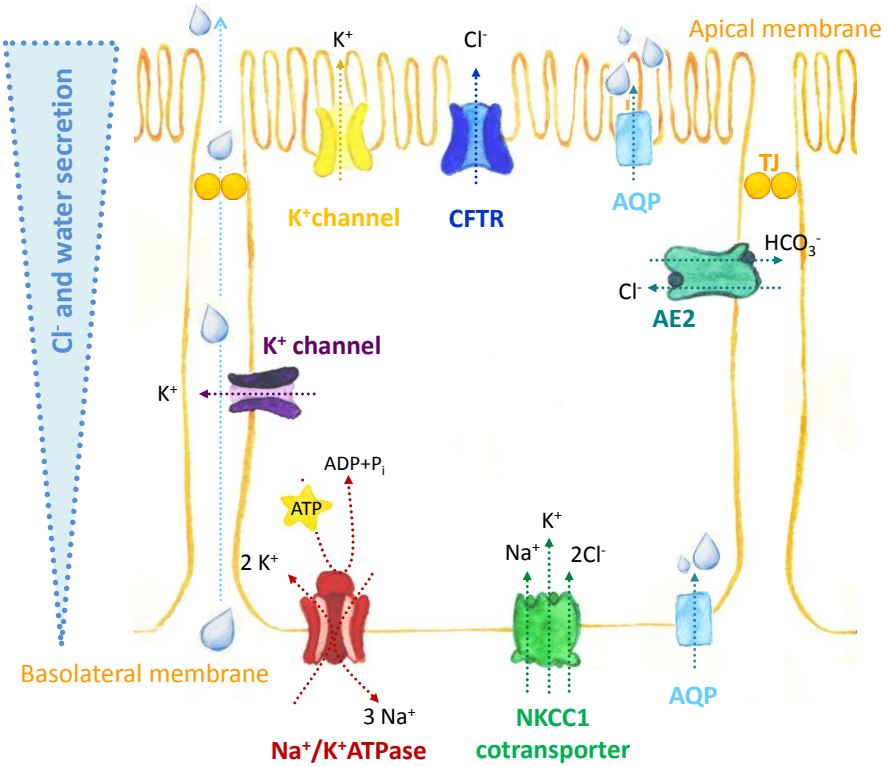


Illustration 3. *Main secretory transporters.*

CFTR is a complex protein belonging to the ATP binding cassette family of proteins (ABC) [93] and is composed of five domains: two membrane-spanning domains (MSDs), two nucleotide-binding domains (NBDs) which bind ATP, and a regulatory (R) domain. The MSDs form the channel pore, phosphorylation of

the R domain triggers channel activity, and ATP hydrolysis by the NBDs controls channel gating. Chloride secretion is unaccompanied by balancing anions and is therefore electrogenic. CFTR may also regulate the activity of other transporters including NHE3 and ENaC (see below) [55, 59]. Water, urea and ATP have also been reported to permeate CFTR Cl^- channels, although the evidence for ATP permeation is controversial [94, 95]. Loss of function mutations in CFTR have profound pathological consequences, which ultimately give rise to cystic fibrosis (CF), an autosomal recessive disease characterized by abnormal airways secretions, chronic endobronchial infection, and progressive airway obstruction [96-98]. While there are other channels capable of mediating apical chloride transport, current evidence suggests that CFTR is by far the most important and nonredundant player *in vivo* [55, 84]. This may also be related to the known ability of CFTR to regulate other ion transporters, as already mentioned [98, 99]. Na^+ ions that enter the enterocyte are extruded by the **Na^+/K^+ ATPase** and also may follow chloride by a paracellular route (if cation movement was impeded chloride movement would be ultimately halted because of the enormous electrical gradient generated).

In addition to chloride, bicarbonate anion is also secreted by the intestinal epithelium using the CFTR exit route and possibly other mechanisms [84]. Bicarbonate is synthesized inside the cell from water and CO_2 by the action of carbonic anhydrase and additionally takenup basolaterally via a Na^+ dependent electroneutral mechanism [55]. K^+ is also secreted via apical channels to a certain extent, particularly in diarrheal states.

2.4. Hydroelectrolytic absorption

Water absorption is linked primarily to Na^+ vectorial transport from the apical to the basolateral membrane of the enterocyte, i.e. from lumen to blood (*Illustration 4. Absorptive transporters*).

Na^+ enters the enterocyte from the apical side via the epithelial brush border membrane (BBM) Na^+/H^+ exchanger 3 (**NHE3** or SLC9A3) predominantly [100]. In mouse models, genetic ablation of NHE3 results in severe intestinal fluid accumulation with subsequent Na^+ diarrhea, net base loss, reduced blood pressure and metabolic acidosis [101], which gives an idea about the main role of this transporter. On the other hand, chloride enters via the SLC26A9 anion antiporter, also known as **DRA** (Down Regulated in Adenoma), in exchange for bicarbonate anion [102, 103]. The central role of DRA in intestinal Cl^- absorption and HCO_3^- secretion is demonstrated by the hereditary disease congenital chloride-losing diarrhea (CLD), an autosomal

recessive disorder characterized by lifelong diarrhea (acidic and salty), hypovolemia and metabolic alkalosis [104].

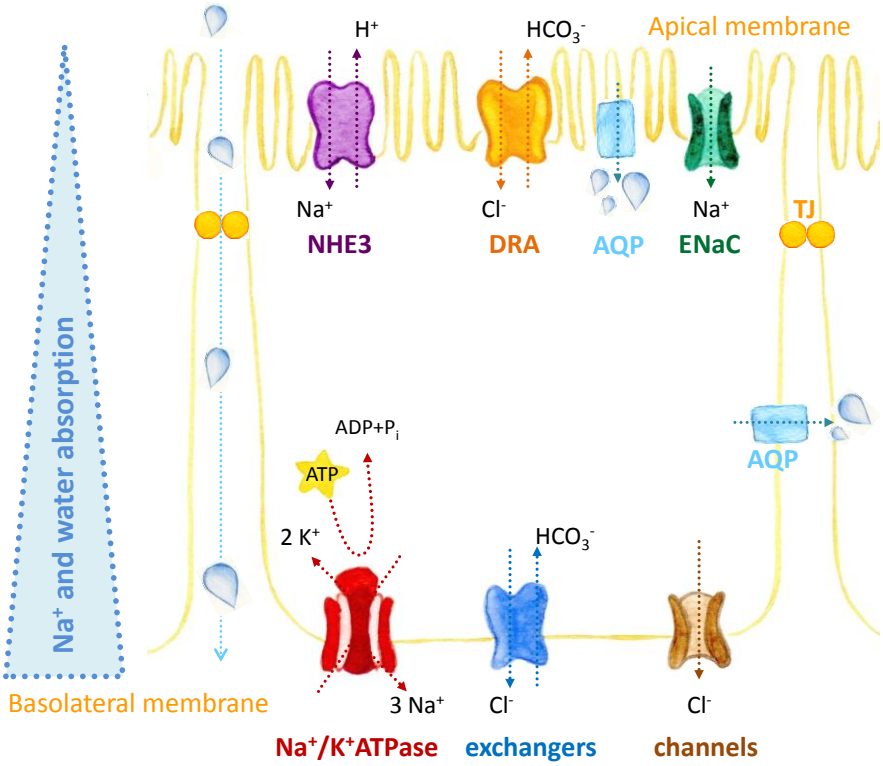


Illustration 4. *Main absorptive transporters.*

Sodium/hydrogen and chloride/bicarbonate exchangers function in a concerted way by intracellular mechanisms that are incompletely understood but seem to involve direct linkage by binding to brush border PDZ domain-containing proteins (members of the **NHERF** and **PDZK1** families) [59, 105, 106]. Because both transporters use a 1:1 stoichiometry, charge distribution at both sides of the membrane is

unchanged and thus transport (NaCl absorption) is said to be electroneutral. The concept that the two exchangers are operationally coupled, thermodynamically and allosterically, by local changes in pH or HCO_3^- [107-109] is consistent with evidence that colonic NaCl absorption is acutely sensitive to changes in HCO_3^- and CO_2 concentrations [107, 110] and depends on carbonic anhydrase activity [111]. Intracellular sodium exits the enterocyte via the **Na^+/K^+ ATPase**, while chloride may exit of the cell by either basolateral channels or exchangers. In the distal regions of the colon, the transepithelial electrical resistance is higher than that of other gastrointestinal segments. Colonic transepithelial voltage (Vt) exceeds -20 mV. In the colon, luminal Na^+ is much lower than in other segments and decreases in a proximal-to-distal manner. The transepithelial Na^+ movement generates a lumen-negative (mucosa-negative) transepithelial voltage faciliting paracellular Cl^- and fluid absorption. Thus, Na^+ absorption also occurs electrogenically via the epithelial sodium channel (**ENaC**), also known as SCNN, composed of α, β and γ subunits. ENaC is induced in the colon descendent by hyperaldosteronism, enhancing Na^+ absorption and favoring a positive Na^+ balance. This action is exerted mainly on the β and γ subunits, (the α subunit is expressed constitutively) [112]. In the colon of $NHE3^{-/-}$ mice, both ENaC and Na^+/K^+ ATPase expression are upregulated. Thus, electrogenic Na^+ absorption by ENaC partly compensates for NHE3 loss of function [100].

There are other transporters that may be involved in hydroelectrolytic absorption. For instance, there are two more NHE transporters: **NHE1** (called SLC9A1), located at the basolateral membrane, and **NHE2** (called SLC9A2), generally expressed alongside NHE3 and considered to contribute to NaCl absorption, intracellular pH and volume regulation [113]. NHE2 and NHE3 are also located at the renal tubule. NHE3 can compensate for defective NHE2 intestinal function [113-115], but NHE2 cannot compensate for loss of intestinal NHE3 function; or SLC26A6 (putative anion transporter-1 **PAT1** or CFEX), one more apical Cl^-/HCO_3^- exchanger, which is electrogenic and may also couple to NHE3 or be activated by the R-region of CFTR [59, 116]. Finally, it is also important to mention a family of Na^+-glucose-linked transporters, called **SGLTs** (also known as SLC5 proteins), several Na^+/amino acid contransporters (SLC6, SLC38, etc.), and other Na^+-coupled solute carriers.

The expression profile of the different absorptive transporters has been shown to be irregular [117]. Thus, NHE3 and DRA do not coexist uniformly along the cecocolonic axis. Animal studies have suggested that in the proximal colon, NHE3 is prominently expressed without DRA, and the mucosal surface pH was found to be uniquely acidic (~6.2), possibly reflecting net H^+ secretion via unpaired Na^+/H^+ exchange. By contrast, in the cecum and distal colon, DRA is strongly expressed without NHE3, and here the mucosal

surface pH was found to be slightly alkaline (~7.5), consistent with net HCO_3^- secretion via unpaired Cl^- /HCO_3^- exchange. The conventional concept of efficient functional coupling between colonic NHE3 and DRA seems possible only within the middle third of the rodent colon, where both exchangers coexist in the same absorptive microdomain. PAT1 is prominently expressed in the proximal small intestine but not in the colon and is therefore unable to cooperate with, or substitute for, DRA in colonic anion transport [118]. These results suggest an unpaired transport activity to maintain an alkaline mucosal surface in the cecum and an acidic mucosal surface in the proximal colon, while coupled NHE3/DRA activities in the midcolon allow for efficient NaCl absorption at a neutral pH on the mucosal surface [119].

Of note, Farkas et al. have obtained evidence in human biopsies in accordance with data from animal models. Thus, in human colonic crypts isolated from human biopsies, electroneutral (via NHE3) and electrogenic Na^+ absorption (via ENaC) are in inverse ratio to each other in the proximal and distal colon of patients, while DRA is more active in the distal colon. There are no differences in the activity of NHE2 [120]. DRA is predominantly expressed in the surface of the epithelium [121].

2.5. Integration of hydroelectrolytic absorption and secretion

Water and ion absorption and secretion occur by several interconnected mechanisms [84] . Therefore the question arises as to how these two opposite phenomena work together. For many years it was believed that absorption and secretion took place in the surface/villus and crypt epithelial cells respectively, particularly in the colon [84]. However, it is now well established that both processes occur across the crypt-surface/villus axis, with the sole exception of electrogenic Na^+ absorption, which is confined to surface epithelial cells in the colon [55]. Thus absorptive and secretory processes take place simultaneously and are regulated concertedly [55, 59, 122].

2.6. Regulation of ionic transport

The regulatory aspects of intestinal ionic transport have been studied extensively. Many physiological and immunological mediators are able to regulate epithelial transport by interacting with specific receptors, including muscarinic [123], substance P [124], epidermal growth factor (EGF) [125], prostaglandins (PGs) [6], serotonin [126], histamine [127], calcitonin gene related peptide [128], interleukin 1β (IL-1β) [12], tumor

necrosis factor (TNF) α [15], platelet activating factor [9] and proteinase-activated receptors [129], among others. In many cases the mechanism is largely indirect, i.e. it involves the participation of additional signaling molecules, like prostaglandins, vasoactive intestinal peptide, nitric oxide, etc. {Rolfe, 1999 #174;Tien, 1991 #175}. The source of these mediators is in many cases extraepithelial, for instance enteric neurons or stromal cells. Epithelial transport can also be affected by exogenous stimuli, including dietary products, biliary acids, bacterial toxins and drugs.

Thus, regulation of epithelial transport in the intestine can be summarized by the endocrine system, the autonomic nerve system, and the immune system. Both enteric nerves and epithelial cells are regulated by sympathetic nerves (proabsorptive effect), parasympathetic nerves (secretory/antiabsorptive effect), the endocrine system and paracrine system (pro- and antiabsorptive effect), and the immune system (secretory effect) [130-132]. Although the mechanisms of enteric nerve–mediated regulation are not fully understood, the end result is mediated predominantly by norepinephrine (proabsorptive effect), somatostatin (proabsorptive effect), acetylcholine (secretory effect), and vasoactive intestinal peptide (VIP) (secretory effect) [133].

In general, intracellular regulation of ionic transport occurs by the Ca^{2+}/IP_3 and cAMP signaling pathways [55], [60].

2.6.1. cAMP and cGMP

3'5'-cyclic adenosine monophosphate, or cAMP, is a second messenger that translates the external stimuli into cell internal stimuli, and is formed from ATP by the action of adenylate cyclase (AC) and which is ultimately degraded to AMP by cAMP phosphodiesterase (PDE) (Illustration *5*).

Most of the effects of cAMP are mediated by protein kinase A (PKA), an enzyme whose inactive form is a tetramer consisting of two catalytic and two regulatory subunits. Binding of cAMP to the two regulatory subunits induces a conformational change that leads to their dissociation from the catalytic subunits. The free catalytic subunits become enzymatically active and are able to phosphorylate serine residues on their target proteins. This triggers a chain of reactions that modulate different signals in the cell. Although most effects of cAMP are mediated by PKA, this second messenger can also directly regulate ion channels and other proteins, independent of protein phosphorylation. An increase of cAMP activates PKA, which in turn

phosphorylates CFTR, augments NKCC1 insertion in the basolateral membrane and activation and opening basolateral K^+ channels (*Illustration 5*). CFTR phosphorylation by PKA results in activation of gating through the destabilization of channel closed states [134]. cAMP also inhibits NaCl electroneutral absorption by interaction of PKA with NHE3 requiring auxiliary proteins [59]. Thus, NHE3 and CFTR display reciprocal inhibition and it has been proposed that CFTR is required for the inhibitory effect of cAMP on NaCl absorption [105, 135]. Modulation of NHE3 activity by cAMP involves primarily changes in turnover number as well as trafficking between the membrane and the endosome recycling compartment.

Cyclic guanosine monophosphate, or cGMP, is also an important second messenger, formed from GTP by guanylate cyclase (GC). An increase in cellular cGMP levels activates CFTR through the action of cGMP-dependent type II protein kinase, and it has been reported to inhibit NHE3 (*Illustration 5*). GC has, as occurs with AC (see below), both membrane-bound and soluble isoforms. The actions of several cells of cGMP and cAMP are clearly differentiated, however, in enterocytes these two second messengers have similar effects.

Ionic transport may be modulated by external

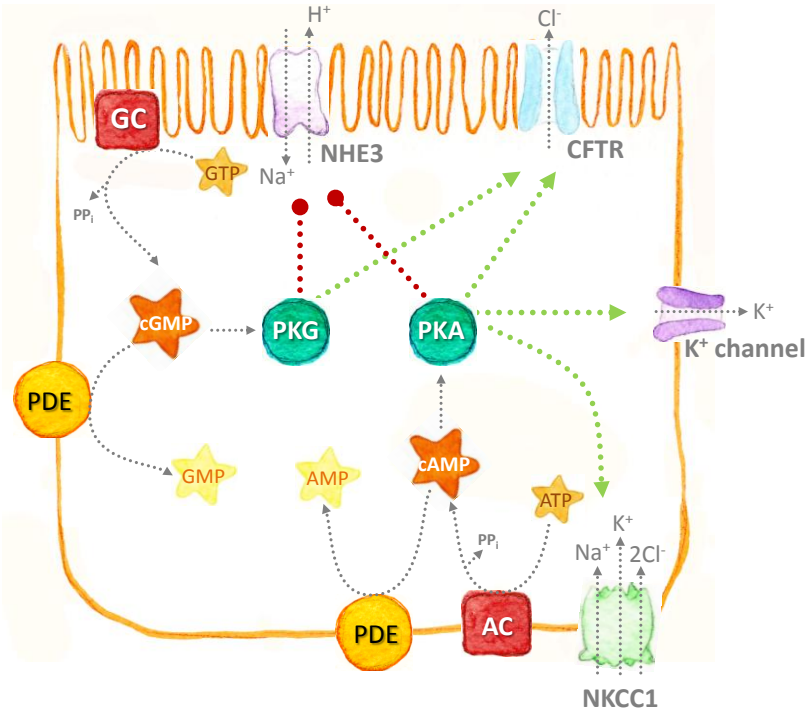


Illustration 5. *cAMP & cGMP pathways in the enterocyte. Positive and negative regulatory influences are shown in green and red respectively.*

agents that interfere with intracellular signaling pathways, for example the phosphodiesterase inhibitor IBMX or the cAMP analogue 8-Br-cAMP (8-bromoadenosine 3',5'-cyclic monophosphate).

2.6.2. Ca^{2+}/IP_3

Another important second messenger involved in regulation of ionic transport is calcium. Phosphatidylinositol 4,5-bisphosphate (PIP_2) is a membrane phospholipid, localized to the inner leaflet of the phospholipid bilayer.

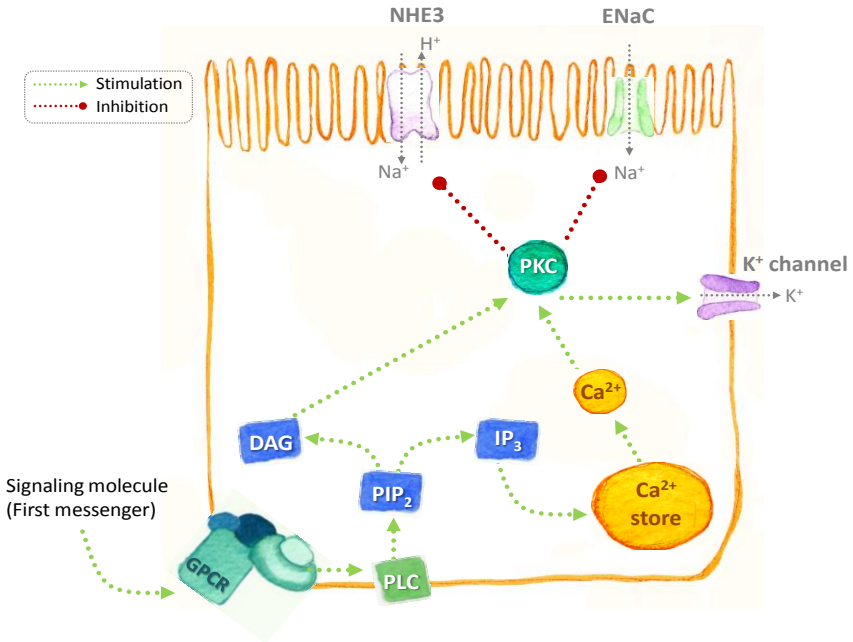


Illustration 6. *Ca^{2+} pathway in the enterocyte. Positive and negative regulatory influences are shown in green and red respectively.*

A variety of hormones and growth factors stimulate the hydrolysis of PIP_2 by phospholipase C (PLC), producing two distinct second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Both stimulate distinct downstream signaling pathways, namely protein kinase C (PKC) and Ca^{2+} mobilization, respectively. IP_3 is a small polar molecule that is released into the cytosol, where it acts to signal the release of Ca^{2+} from intracellular stores. When intracellular Ca^{2+} is increased, the main regulatory mechanism is activation of basolateral K^+ channels, augmenting the electrical gradient and favoring anion secretion (*Illustration 6*). An important issue is that CFTR is not activated by the Ca^{2+} pathway *in vivo* [55]. Extensive evidence obtained in humans and animal models indicates that Ca^{2+} driven Cl^-/HCO_3^-

secretion depends on the independent activation of CFTR by mediators operating via cAMP/cGMP, such as prostaglandins [136, 137]. Since prostaglandins are basally produced *in vivo*, this represents no obstacle as long as CFTR is operative, i.e. in individuals not suffering from cystic fibrosis. There is evidence pointing at the existence of Ca^{2+} activated apical channels in the intestinal epithelium but CFTR is generally considered by far the most important exit pathway for anions in enterocytes, at least *in vivo* [55]. Intracellular Ca^{2+} increase also inhibits NHE3 via PKC, and it reduces ENaC mediated Na^{+} uptake in the distal colon [59].

2.6.3. Nitric Oxide

Nitric oxide (NO) is one of the smallest and simplest biologically active molecules, with an important signaling role in the gastrointestinal tract [138]. Potential sources of this free radical in the gut include: intrinsic intestinal tissue (mast cells, epithelium, smooth muscle, and neural plexus), resident and/or infiltrating leukocytes (neutrophils, monocytes), reduction of luminal gastric nitrate, and denitrification by commensal anaerobes [139, 140]. Besides eukaryotic NO production, it is well known this gas can be produced also by microorganisms. In biological systems, NO has a half life of less than 5 seconds, rapidly degrading to nitrite (NO_2^-) and nitrate (NO_3^-) in the presence of oxygen and water [141]. Being soluble in both water and lipids, it freely traverses cell membranes and passes into adjacent target cells [142]. NO can be synthesized in eukaryotic cells through the oxidation of L-arginine by the action of a stereospecific group of enzymes called nitric oxide synthases (NOS) [143, 144]. The neural and endothelial isoforms of NOS (nNOS and eNOS) are expressed under resting conditions, whereas inflammatory stimuli are required for the induction of the inducible type (iNOS). Under resting conditions, mucosal perfusion is regulated by NO derived from the vascular endothelium of the mesenteric bed. NO can be produced by enterocytes through both isoforms, constitutive and iNOS [145, 146]. NO may protect the gastrointestinal mucosa from a variety of stimuli (caustic ingestion, ischemia, ischemia/reperfusion injury, early endotoxic shock) by maintaining mucosal perfusion, inhibiting neutrophil adhesion to mesenteric endothelium [147], blocking platelet adhesion [148], preventing mast cell activation and down-regulating nuclear factor-kappa B (NF-κB) [149]. Coordination of peristalsis action is mediated by the release of NO, which acts as the principal neurotransmitter of the nonadrenergic, noncholinergic enteric nervous system.

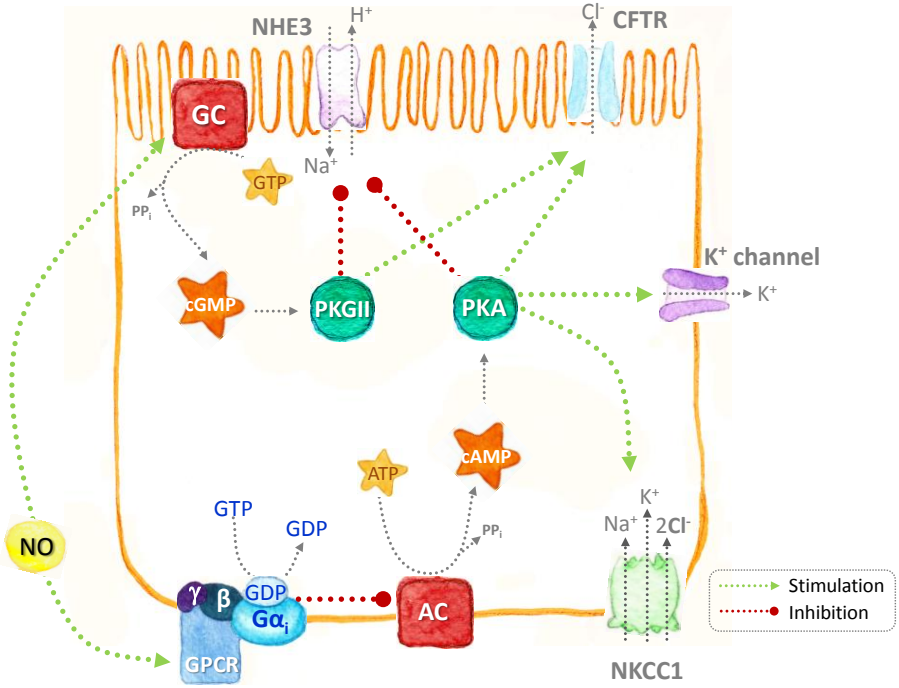


Illustration 7. *NO in the enterocyte. Positive and negative regulatory influences are shown in green and red respectively.*

Thus, NO can be protective. But it may be cytotoxic as well. Excessive NO may directly injure the mucosa. Barrier function of the intestinal mucosa is protected by NO in the early stages of injury, when neutrophil adhesion, ischemia, and mast cell activation are relevant. Inhibition of NO synthesis ameliorates barrier dysfunction during more advanced stages of inflammation, when activation of iNOS yields toxic concentrations of NO. At high concentrations, NO reacts with superoxide anions and peroxinitrite is formed, which is a strongly toxic oxidant with several reaction pathways such as disrupting the actin cytoskeleton [150], inhibiting ATP formation, dilating cellular tight junctions, and producing a hyperpermeable state [151].

NO production is increased in IBD [152] but whether NO acts primarily aggressively or protectively in IBD is still unclear [153]. The induced overproduction of NO is likely to be responsible for the decreased motility in colitis where NO is suggested to exert a suppressive tone on colonic contractility, which is reversed by blockade of NO synthase. This radical has been considered a regulator of basal intestinal water transport and a mediator of pathological conditions. Studies suggest that NO may interfere with cAMP-regulated secretion, including CFTR trafficking, by inhibiting AC in a guanylate cyclase-independent fashion [30]. Thus, NO may exert inhibitory effects on intestinal secretion acting by direct and indirect mechanisms (*Illustration 7*). Thus, studies suggest that physiologically NO promotes fluid absorption, but in pathophysiological states it may be produced in high concentrations capable of evoking net secretion. This is why until that time, it remains to be seen whether NO is a mucosal ´friend´ or ´foe´.

2.6.4. Summary of the regulation of CFTR, NHE3 and DRA by second messengers

Briefly, when the intestinal mucosa (apical or luminal surface) is stimulated by enterotoxins, neurotransmitters, or drugs, increasing intracellular cAMP [154, 155] , Ca^{2+} [154, 156, 157], or cGMP [158], electroneutral NaCl absorption is inhibited, and electrogenic Cl^- secretion is activated [56]. The inside negative electric potential of epithelial cells provides the driving force to secrete Cl^- and water. NHE3 is another main target of these second messengers. Its inhibition requires both second messenger–activated protein kinases and the NHERF scaffold proteins. The phosphorylation-mediated internalization of NHE3 seems to be the dominant mechanism for NHE3 inhibition by second messengers.

Conversely, activation of NHE3 occurs by decreased intracellular pH (pHi) or increased cellular metabolism (e.g., glucose transport and decreased pHi) [133, 159]. It has been shown that DRA and PAT1 also activate the Cl^- secretion by CFTR after the stimulation of the intestinal mucosa [160]. In contrast to NHE3 regulation, the inhibitory mechanism of DRA and PAT1 is poorly understood [133].

2.6.5. Other regulatory aspects

- **PDZ adaptors**

Protein–protein interactions play a key role in the regulation: several transporters in epithelial cells interact with the so-called adaptor proteins, which are membrane anchored and interact with both transporters and other plasma membrane proteins, receptors, and intracellular signaling molecules. Among them, four PDZ (PSD95/Dlg/ZO1) adaptors, PDZK1 (also known as NHERF3), PDZK2 (also named NHERF4), sodium/proton exchanger regulatory factor (NHERF) 1, and NHERF2 interact with the C-terminus of transporters expressed in intestinal epithelial cells [106].

The PDZ adaptors are thus involved in regulation of signal transduction, and can stabilize the transporters at the cell surface. Roles of PDZK1 as an adaptor have been demonstrated for various transporters in the small intestine, for example: forskolin-responsive intestinal net Na^+ absorption was significantly reduced in *pdzk1*$^{-/-}$ mice, even though the expression level and localization of NHE3 were not significantly different from those of wild-type mice [161]. Another example is the study in *nherf1*$^{-/-}$ mice, where the expression of NHE3 and CFTR on brush-border membranes of epithelial cells and in crypt cells, respectively, is reduced [162, 163]. Levels of NHE3 and NHERF1 were significantly lower in mucosal biopsies from patients with inflammatory bowel disease (IBD) as well as from acute murine IBD models, suggesting that downregulation of NHERF1 induces IBD-associated diarrhea, possibly caused by unbalanced ion concentrations [164].

In the small intestine, absorption of NaCl and nutrients is mediated mainly by villus cells, where secretion of fluid containing Cl^- and HCO_3^- is mediated mainly by crypt cells [165, 166]. But at the same time, it is also believed that electroneutral NaCl absorption and electrogenic Cl^- secretion occur in the same cells. It has been proposed that this can be explained only because the cross-regulation of NHE3, DRA and CFTR by the PDZ adaptor proteins [133].

- **Basolateral transporters and homeostasis**

It is worth mentioning in the regulation of ionic transport, the role of basolateral electrolyte transporters in colonocyte homeostasis [167]. Early on, Na^+/H^+ exchange was implicated in 'housekeeping' functions such as pH_i and cell volume regulation, which are particularly important in cell types with a high secretory activity that are prone to disturbances in pH and cell volume. Furthermore, the colonocyte is exposed to high intraluminal concentrations of short chain fatty acids (SCFA) (up to 100 mM) and $NH4^+$ (up to 20–25 mM) [168, 169]. Therefore, keeping intracellular pH within an optimal range is crucial for colonocyte metabolism and therefore cell survival. In particular, NHE1, but also other NHE isoforms and Cl^-/HCO_3^- exchangers, Na^+/HCO_3^- cotransporters, $Na^+/K^+/2Cl^-$ cotransporters, and others have been implicated in this process [170]. Furthermore, secretion causes volume loss, which in turn leads to a regulatory volume increase for the cell to stabilize its shape, subcellular structure and concentration of cytoplasmatic components, and this process involves both NHE isoforms as well as DRA and NKCC1 [171].

Before we start to elucidate the consequences of the alteration of ion transport, there is one last point important to describe: our previous results indicate a crucial role of the adenylate cyclase / cAMP pathway in inflammation. This is why the description of this enzyme and it role in the production of cAMP and ion transport is a key in our project.

2.7. Adenylate cyclase

The mammalian Adenylate Cyclase (AC) is an integral membrane family of proteins composed of two hydrophobic domains (with six transmembrane spans) and two cytoplasmic domains (C1 and C2), the catalytic site, resulting in a pseudosymmetrical protein. The hydrophobic domain topology is reminiscent of the ABC family of transporters such as CFTR [172]. The catalytic cytosolic regions of mammalian ACs also share significant sequence similarity to the corresponding regions of soluble guanylate cyclase (GC) [173].

As mentioned above, all ACs convert the intracellular adenosine triphosphate (ATP) to cAMP and pyrophosphate. The activation of AC is initiated by the binding of hormones to cell surface receptors of the G-protein-coupled type (GPCR) [174]. Prostaglandins, epinephrine, dopamine, adenosine, and glucagon are a few examples of the many hormones that activate AC through GPCR (*Illustration 8*). AC is stimulated by

ligands-bound receptors by way of an intracellular, membrane-associated heterotrimeric G protein [175] composed of a guanosine diphosphate (GDP)–bound α-subunit and an obligate βγ heterodimer. Hormone-dependent activation of receptors leads to the exchange of GDP for guanosine triphosphate (GTP). Conformational changes due to GTP binding result in the dissociation of the heterotrimeric G protein into α and βγ subunits, which then interact with their respective effectors.

There are multiple classes of α -subunits that regulate AC, either in a stimulatory ($G\alpha_s$ family), or inhibitory ($G\alpha_i$ family) manner (*Illustration 8*) [175]. The two Gα families are normally coupled to distinct receptor subtypes, and may manifest selectivity for given AC isoforms. The βγ-subunits also regulate AC, in an AC subtype–specific manner [176, 177]. Additionally, calcium ions are very strong modulators of some isoforms of AC [178-181]; thus, G proteins that regulate calcium entry through voltage-dependent Ca^{2+} channels may also regulate AC activity [182-184]. Also a number of other proteins like the protein associated with Myc-PAM, *Escherichia coli* protein SlyD, protein kinases (PKA, PKC, and calmodulin (CaM) kinase), phosphatases (calcineurin), or small molecules such forskolin, pyrophosphate or adenosine and cAMP analogs, have been shown to interact directly with ACs, but their biological significance has yet to be determined. Finally AC can be regulated by posttranslational modification, including phosphorylation, glycosylation and S-nitrosylation.

There are different classes of ACs in mammalian cells, from AC1 to AC9, plus the soluble AC (sAC) [185].

Thus, all the ACs included from AC1 to AC9 are G protein-regulated transmembrane adenylate cyclases (tmACs), and are not regulated by bicarbonate [176, 177, 186]. They share a large sequence homology in the primary structure of their catalytic sites and the same predicted three-dimensional structure [185]. However, at the protein level, there are many proteins and small molecules that affect the catalytic activity of ACs. So they can be divided into 4 distinct families based on their amino acid sequence similarity and functional attributes (*Illustration 8*):

- The Ca^{2+}–CaM-sensitive forms are types AC1, AC3, and AC8.
- The Gβγ-stimulated forms are represented by AC2, AC4, and AC7.
- AC5 and AC6 are distinguished by their sensitivity to inhibition by both Ca^{2+} and Gαi isoforms ($G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_z$).

- AC9 is the most divergent of the membrane-bound family and is highly insensitive to the diterpene forskolin.

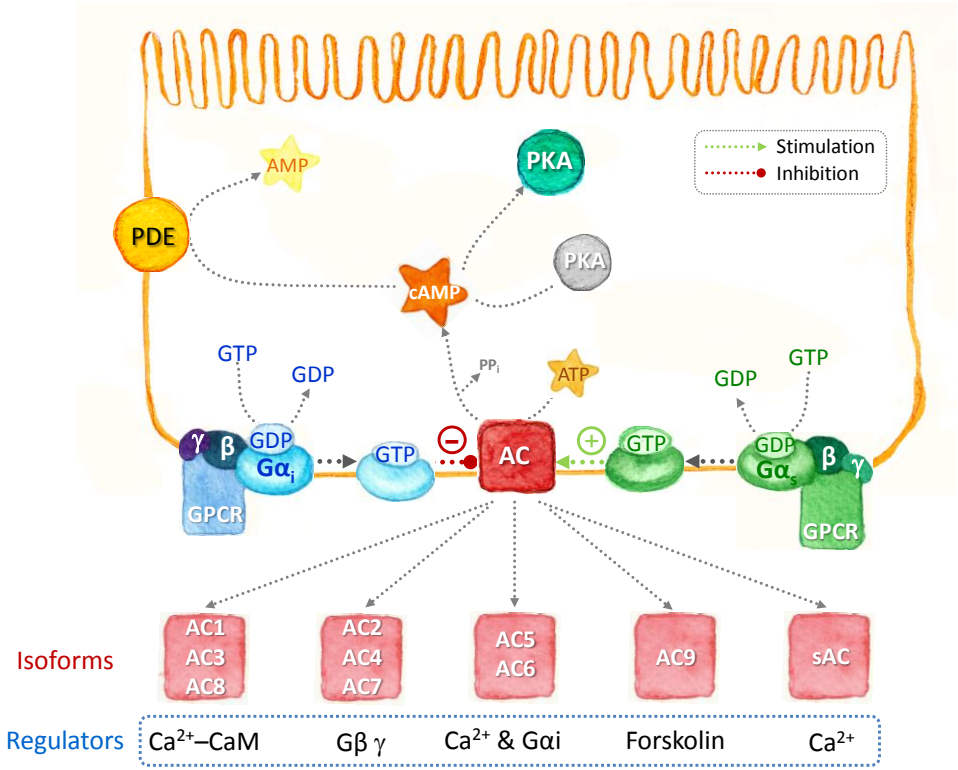


Illustration 8. *Adenylate cyclase regulation, isoforms and regulators. Positive and negative regulatory influences are shown in green and red respectively.*

Finally, The last AC isoform, the calcium-regulated soluble adenylate cyclase (sAC), is the most divergent of all the mammalian cyclases, and is similar to cyclases found in cyanobacteria [187]. The broad distribution of AC isoforms suggests that any given cell contains multiple isoforms. Thus, depending on the

properties and the relative levels of the isoforms expressed in a tissue or a cell type, extracellular signals received by the GPCR can be differentially integrated.

The biochemical assessment of the ACs has revealed several regulatory pathways that control AC activity. In contrast, a number of factors have resulted in a relative paucity of physiological data describing these complex regulatory pathways. The most notable obstacle is the multiplicity of the isotypes expressed within a given cell type, further complicated by the varying effects of modulators, as well as the particular intracellular milieu. Several studies in cell transfections and animal models show an important role of the different ACs isoforms in the brain function-learning and memory [188-195], olfaction [196], sperm development [197], acute responsiveness and tolerance to morphine (36), hearth [198, 199], cell differentiation [200] and human diseases like overactive thyroid adenomas and non-autoimmune autosomal dominant hyperthyroidism [201], endocrine tumors, McCune-Albright syndrome and testitoxicosis [202]. Cholera toxin, for instance, produces a covalent modification of the α_s subunit of the G protein. Thus AC is permanently activated and produces copious amounts of cAMP, the result being continuous CFTR dependent chloride and water secretion, which may be as high as 20 l in a day.

Finally, AC sensitization has to be mentioned. It has long been appreciated in model systems for drug abuse (like opiates), withdrawal, and recovery [203]. This phenomenon, also called cAMP overshoot [204], is characterized by an enhanced responsiveness to drug-stimulated cAMP accumulation following persistent activation of $G\alpha_{i/o}$-coupled receptors.

AS is already mentioned, there are several modulators of the cAMP synthase, which activate AC through GPCR. We have special interest in one of them because our studies: the endogenous opioids.

2.8. Opioid receptors

There are three main opioid receptors, whose name comes from the different anatomical location and pharmacological profiles of compounds that were eventually used to name them: δ (delta-vas deferens), μ (mu- morphine), and κ (kappa-kerocyclazocine).

Recently, a fourth opioid like receptor has been included in the opioid receptor family and is termed the nociception orphanin FQ peptide receptor. Receptor nomenclature from the International Union of Pharmacology (IUPHAR) opinion is MOP (mu), KOP (kappa), DOP (delta) and NOP for the nociception orphanin FQ peptide receptor [205] All four are GPCRs sharing seven transmembrane topology. The greatest diversity is found in their extracellular loops. The different receptors are widely distributed in brain and spinal cord because it diverse effects on central nervous system. In addition, these receptors are expressed in a wide variety of peripheral tissues, including vascular, cardiac, airway/lung, gut, and many resident and circulating immune/inflammatory cells [206].

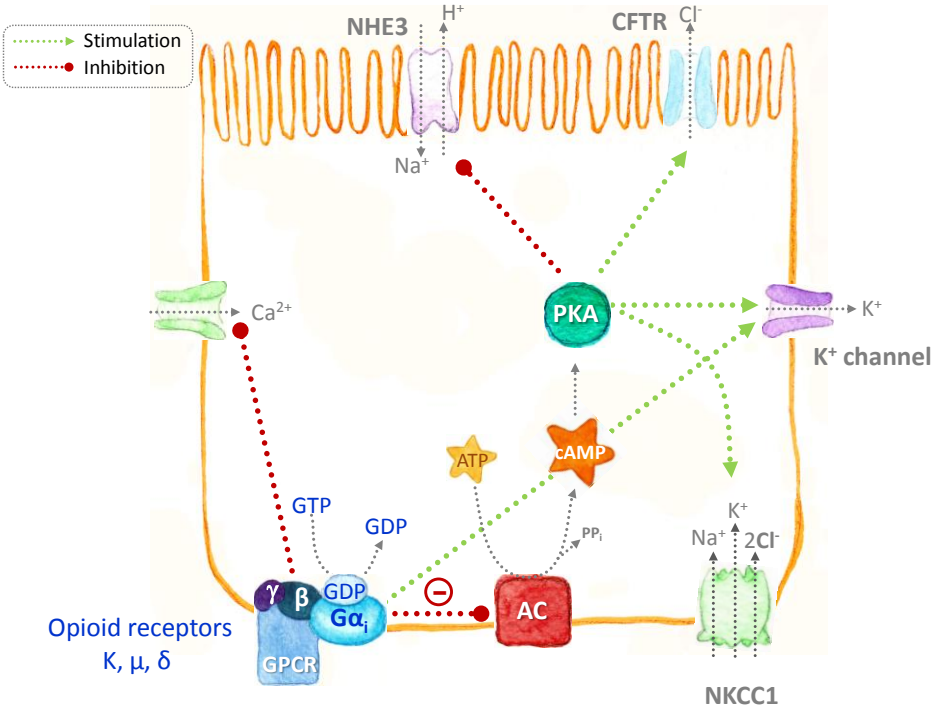


Illustration 9. *Opioid receptors. Positive and negative regulatory influences are shown in green and red respectively.*

Opioid receptors couple to inhibitory G-proteins (*Illustration 9*). Activation of opioid receptors results in conformational changes in the GPCR, initiating the G protein activation/inactivation cycle. It leads to:

- Reduced cAMP production via inhibition of G_i protein, which inhibits AC.
- Closing of voltage sensitive Ca^{2+} channels.
- Stimulation of K^+ efflux, probably because the activation of the $\beta\gamma$ subunits, and leading to hyperpolarization.

Overall, this results in reduced neuronal cell excitability leading to a reduction in transmission of nerve impulses along with inhibition of neurotransmitter release.

After reviewing the most important modulators of ionic transport, it is important to remark that there is a delicate balance between ion absorption and ion secretion along the intestine. This delicate balance can be upset giving rise to diarrhea.

3. ALTERATIONS IN INTESTINAL TRANSPORT: Diarrhea

Inflammation has a profound impact on intestinal physiology, as it can affect gut motility, neuronal functionality and hydroelectrolytic transport [1-3], giving rise to diarrhea. Worldwide, diarrhea remains the second leading cause of death of children under five years old and accounts for 15% of childhood deaths worldwide [207]. Diarrhea is primarily a symptom rather than a disease and may occur in many different conditions. It may be loosely defined as the fecal excretion which is too frequent and/or too watery and exceeds a stool weight of more than 200 g/d [208]. It should be noted that diarrhea may or may not be caused by an inflammatory condition, other major culprits being malabsorption, bacterial toxins, viral and protozoal infection and laxatives. Since diarrhea is naturally associated with increased bowel movements and sometimes with urge to defecate, motility disturbances have long been suspected to play a predominant role in this condition in general. It is becoming increasingly evident however that isolated motility alterations (i.e. in the absence of luminal fluid accumulation) rarely cause diarrhea. Rather, although uniformly present, increased intestinal motility is a natural consequence of bowel wall distention, which is expected when water accumulates in the lumen.

The term diarrhea is used popularly as a synonym of “colitis”. Nevertheless, this term implies inflammation and most cases of diarrhea are of non-inflammatory and acute nature. It is important to differentiate between acute and chronic colitis:

- Acute colitis of any etiology is associated with inhibition of ion (NaCl) absorption and increased Cl^- and HCO_3^- secretion, alterations in tight junction function and regulation, and activations of the NF-κB and mitogen-activated protein kinase pathways (via Toll-like and cytokine receptors), all resulting in water accumulation in the large bowel and diarrhea. These episodes are relatively often and typically are due to bacterial or viral infections or to parasitic infestations (e.g. *Giardia*), which typically are self-limiting or can be effectively controlled with medication [207]. It is important to note that inflammation may be minimal or being entirely absent in such episodes. Persistent diarrhea (>2 weeks) raises the question of a protozoal infection [209]. Even these infections frequently abate within one month of onset. A duration of four weeks or more has been set arbitrarily as the threshold duration for a diagnosis of chronic diarrhea [209]. Although some

infections, such as those due to *Clostridium difficile*, *Aeromonas* or *Yersinia*, may last longer than four weeks, most chronic diarrheas will be found to have a noninfectious cause.

- Conversely, chronic colitis (liquid stools lasting more than four weeks) is a more restricted phenomenon which has different causes [4]. As in acute cases, chronic colitis is typically accompanied by diarrhea, but the mechanisms are somewhat different, and as we will see, the 'chronic picture' is more complex. Illnesses that course with chronic colitis are, among others:

    - Inflammatory bowel disease (IBD): is an inflammatory disorder of the gastrointestinal tract that encompasses two idiopathic and major inflammatory diseases: Crohn's disease (CD) and ulcerative colitis (UC). This condition will be described in detail in the next section.

    - Microscopic colitis: is characterized by a normal macroscopic appearance, while microscopic examination reveals different abnormalities [210].

    - Pseudomembranous colitis: is manifested as inflamed mucosa and characterized by the presence of yellow-white plates with pseudomembranous appearance, related to the presence/overgrowth of *Clostridium difficile* [211]. However, the pseudomembranes are present on top of the mucosa, and do not result in ulceration of the underlying tissue like in IBD.

    - Infectious colitis: although as stated above, microorganisms do not generate in general chronic diarrhea, in some cases it can happen. This colitis may cause mucosal inflammation and look identical to IBD. Histological findings may help to distinguish these disorders, with crypt distortion favoring the diagnosis of IBD [212].

    - Non-steroidal anti-inflammatory drug-induced colitis: the chronic use of nonsteroidal antiinflammatory drugs (NSAIDs) has been associated with a variety of pathologic changes throughout the GI tract. Thus, NSAID use should be considered in the differential diagnosis of ulcerations and/or inflammatory changes. In addition, NSAIDs favor the exacerbation of preexisting IBD.

    - Radiation colitis: radiation can cause mucosal inflammation that resembles UC. It tends to be continuous, friable, and left-sided. A history of abdominal radiation, even if temporally distant, should differentiate the two diagnoses.

- Ischemic colitis: this kind of colitis is due to insufficient blood flow to maintain metabolic demand in the gastrointestinal tract. Ischemic colitis tends to be continuous, left-sided, and associated with mucosal friability, findings that resemble UC. The keys to proper diagnosis are sparing of the rectum in ischemic colitis and the presence of risk factors for ischemic colitis.
- Colitis associated with diverticular disease: infrequently, patients with diverticular disease develop a segmental colitis, most commonly in the sigmoid colon. The clinical, endoscopic, and histological features vary but can resemble IBD.

Before we continue to discover this complex picture, let us describe IBD with just a few strokes off the brush.

4. INFLAMMATORY BOWEL DISEASES

4.1. Generalities

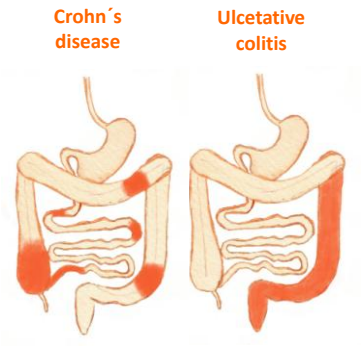


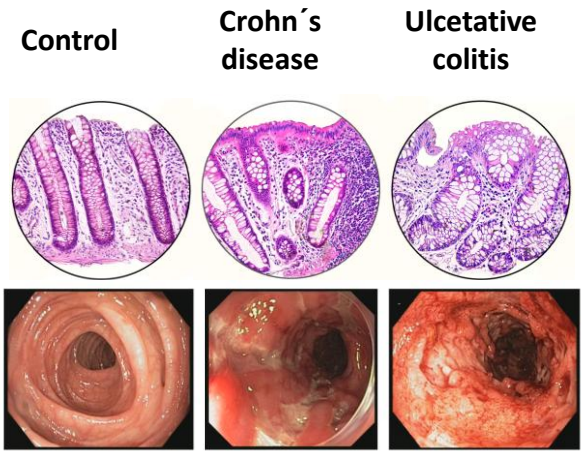
***Illustration 10**. Anatomic distribution of normal, CD and UC intestine: colon.*

Inflammatory Bowel Disease (IBD) is a chronic relapsing inflammatory disorder of the gastrointestinal tract that encompasses two idiopathic and major inflammatory diseases: Crohn's disease and ulcerative colitis [4]. Both forms of IBD significantly impair quality of life, and require prolonged medical and/ or surgical interventions. What makes it particularly challenging is its still unknown cause, its unpredictable presentations and symptoms, the less than optimal treatments, and a continuous rise in its incidence and prevalence in many areas of the world. CD and UC share many epidemiological, pathological and therapeutic characteristics. And both have overlapping symptoms, including abdominal pain, vomiting, diarrhea, fever, hematochezia and weight loss. This is why in some patients, it is not possible to distinguish which form of IBD is present (indeterminate colitis). There are, however, important differences that distinguish these inflammatory disease processes,

according with the location of the inflammation, appearance and immunological profile [213, 214]. A summary is given in *Table 1 and 2*.

- Location of inflammation: In CD, the inflammatory process may occur anywhere along the digestive tract, from the mouth to the anus *(Illustration 10)*. Actually, in about 50% of the patients, the terminal ileum is involved, in about 30% of the patients the disease is located in both the ileum and colon and in approximately 20% the disease is limited to the colon. While in UC the disease often involves the rectum and extends proximally, but remains restricted in the mucosal layer of the colon (although in some patients, especially pediatric ones the entire tract may be involved).

- Appearance *(Picture 2)*: CD is characterized by transmural inflammation and by skip lesions. The colonic wall may be thickened and, because of the intermittent pattern of diseased and healthy tissue, it may have a "cobblestone" appearance. Typically in UC, the colonic wall is thinner and shows continuous inflammation involving the mucosal layers and occasionally the submucosa with no patches of healthy tissue in the diseased section. UC is more often characterized by gastrointestinal bleeding. Radiographic studies of patients with CD characteristically show fistulas, abdominal abscesses, asymmetry and ileal involvement. In contrast, radiographic studies of patients with UC show continuous disease without fistulizing or ileal disease.



***Picture 2**. Histology (H&E) and endoscopic views of normal, CD and UC human intestine.*

- Immunological Profile: Traditionally, as the Th1/Th2 paradigm was proposed, it was established that CD differed from UC in that CD seemed to be a Th1 cytokine-mediated disease while UC seemed to be a non typical Th2 type-like cytokine profile, but this view is probably oversimplistic.

As will be discussed later, the disease cannot be considered as an imbalance between Th1 and Th2 response, but the consequence of an exacerbated Th effector response.

DIFFERENCES	CROHN'S DISEASSE	ULCERATIVE COLITIS
Location	Inflammation may occur anywhere along the digestive tract, from mouth to anus	Inflammation limited to the **mucosal layer of the colon**
Inflammation	Asymmetrical inflammation, in patches	Continuous inflammation of the colon
Appearance	Colon wall may be thickened and may have a cobblestone appearance	Colon wall is thinner and shows continuous inflammation
Depth of inflammation	Ulcers along the digestive track are deep and may extend into all layers of the bowel wall	Inflammation only affects the mucosa layer
Bleeding	Not common	Bleeding from the rectum during bowel movements

***Table 1**. Differences between Crohn's disease and ulcerative colitis.*

Symptoms of Crohn's Disease	Symptoms of Ulcerative Colitis
• Abdominal pain, cramping or swelling • Anemia • Fever • Gastrointestinal bleeding • Joint pain • Malabsorption • Persistent or recurrent diarrhea • Stomach ulcers • Vomiting • Weight loss	• Abdominal pain or discomfort • Anemia caused by severe bleeding • Bloody diarrhea • Dehydration • Fatigue • Fever • Joint pain • Loss of appetite • Malabsorption • Rectal bleeding • Urgent bowel movements • Weight loss

***Table**2. Differences between Crohn's Disease and Ulcerative Colitis*

The severity of disease in patients with IBD is important in guiding clinical management and can predict long-term outcomes. It can be objectively measured using a clinical disease activity index, the Montreal

classification. Thus, UC can be classified in clinical remission, mild, moderate and severe colitis. The classification for CD encompasses age at diagnosis, location and behavior [215].

4.2. Systemic and extra-colonic manifestations of IBD

At least 25% of IBD patients develop extraintestinal manifestations of some kind. Some of these entities are associated more with CD than UC, including articular manifestations, oral lesions, clubbing, gallstones, pancreatitis, and renal and urologic conditions such as calcium oxalate stones, cystitis secondary to enterovesical fistulae, and amyloidosis. Other extraintestinal findings, including skin and eye disorders, are seen with equal frequency in both CD and UC and may dominate the clinical presentation. The pathogenesis of extraintestinal manifestations is incompletely understood [4].

- Articular manifestations: This is the most common extraintestinal manifestation of IBD, which occurs in 6-25% of patients. There are three general patterns of arthritis in IBD patients: peripheral arthritis, ankylosing spondylitis and sacroileitis, which differ in joint distribution, relation to IBD activity, and joint destructiveness. Thus, the arthritic symptoms may appear before IBD and do not necessarily follow the course of the intestinal disease. 20% of patients with UC have peripheral arthritis, which affects large, weight-bearing joints such as knees or ankles. Arthritis signs and symptoms usually accompany exacerbations of IBD.
- Skin manifestations: A variety of cutaneous lesions may be seen in association with IBD, occurring in 9-20% patients. Erythema nodosum and pyoderma gangrenosum are commonly associated with this disease [216]. Other dermatological sequelae include dermatitis, erythematous rash, psoriasis, urticaria, pityriasis, lupus erythematosus, vitiligo, Sweet syndrome and ecchymosis. Some lesions respond to treatment of bowel disease, but others do not.
- Ocular manifestations: About 5% of patients with IBD present ophthalmologic manifestations and may include uveitis, episcleritis and keratoconjunctivitis. Symptoms of these complications include headache, photophobia, blurred vision, burning and increased secretions from the eyes. Ocular lesions usually occur when the bowel disease is active, but this is not absolute [217].
- Bronchopulmonary diseases: Pulmonary complications of IBD, although uncommon, include airway inflammation, parenchymal lung disease, serositis, thromboembolic disease, and drug-induced lung toxicity. In patients with IBD, inflammation of the trachea, bronchi, and bronchioles may develop and

rarely preceding the onset of IBD. Bronchiectasis is the most common form of airway involvement, but subglottic stenosis, airway-enteral fistulae, bronchitis, and bronchiolitis also occur. Several patterns of diffuse lung parenchymal involvement have been described in IBD, including organizing pneumonia, eosinophilic pneumonia, and nonspecific interstitial lung disease.

- Other manifestations: IBD can also be associated with a variety of hepatobiliary disorders, renal dysfunctions, hematologic and cardiovascular complications, osteoporosis and extra-intestinal cancer [4, 218-220].

In most situations, extraintestinal manifestations respond to standard medical therapy. On rare occasions, a total proctocolectomy may be necessary to control severe extraintestinal manifestations of this disease.

4.3. Epidemiology

Multiple studies have evaluated the epidemiology of IBD in various geographic regions. Epidemiological studies have shown that IBD has a combined prevalence of 300-400 cases per 100,000 people in northern climates of well-developed areas, such as North America and northern Europe and incidence rates ranging from 3-14 cases per 100,000 people [221, 222]. The result prevalence and incidence is the lowest in southern climates and underdeveloped countries, such as South America, Southeast Asia and Africa [223], but a rapid increase has been observed in some of these regions as well, especially in Brazil and China. This variation in incidence rates by geographic location may be the result of environmental factors, such as industrialization, sanitation, hygiene and access to specialized health care [223]. The prevalence and incidence rates also differ in different racial (e.g. African American, Asians, Hispanics, Caucasians) and ethnic status (e.g. Jewish vs. non-Jewish) [224, 225], implicating an important role for environmental factors as well as genetic influences.

Seasonal variation in flares of IBD (with peaks in the spring) has been suggested in some reports; however, the magnitude of the association, if any, is weak, and discordant data have also been published [226-238].

The incidence of IBD may have changed over time [239-243]. The incidence of CD appears to have leveled off, and is now approximately equivalent to UC in North America and Europe.

On the other hand, there is also a relationship between age and IBD. The age of onset of most cases of UC and CD have their greatest onset in the adolescent and young adult years. Most studies show a peak between ages 15 and 25. Many studies suggest a bimodal age distribution for both disorders with a second peak between 50 and 80 years [238]. It is not clear whether this second peak relates to greater susceptibility to disease with increased age, the late expression of an earlier environmental exposure, or misdiagnosis of ischemic colitis as IBD.

Finally, there appear to be small differences in IBD incidence by gender. In general, there is a slight female predominance in CD, although in some low incidence areas a male predominance exists. The female predominance, especially among women in late adolescence and early adulthood, suggests that hormonal factors may play a role in disease expression. In contrast, there may be a slight male predominance in UC [244, 245].

4.4. **Etiology**

IBD is considered as an idiopathic and multifactorial disease. Over the years many theories have been proposed to explain IBD pathogenesis, ranging from infectious to psychosomatic, social, metabolic, vascular, genetic, allergic, autoimmune and immune-mediated. There is now a general consensus that IBD is the result of the combined effects of four basic components: global changes in the environment, the input of multiple genetic variations, alterations in the intestinal microbiota, and aberrations of innate and adaptive immune responses. There is also agreement on the conclusion that none of these four components can by itself trigger or maintains intestinal inflammation but it is their integration and reciprocal influence what determines whether IBD will emerge and with which clinical phenotype.

Coexistence of these factors causes an unregulated immune response at the intestinal mucosa, resulting in a chronic intestinal inflammation.

4.4.1. Genetic predisposition

Multiple genetic variations have been implicated in the pathogenesis of IBD. Approximately 10 to 25 % of individuals with IBD have a first degree relative with either CD or UC [246-248]. Although the genetic component is stronger in CD than in UC, and despite their distinct clinical features, approximately 30% of these IBD-related genetic loci are shared between both conditions, indicating that these diseases engage common pathways [249, 250]. Analyses of the genes and genetic loci implicated in IBD show several pathways that are crucial for intestinal homeostasis, including barrier function, epithelial restitution, microbial defense, innate immune regulation, reactive oxygen species generation, autophagy, regulation of adaptive immunity, endoplasmic reticulum stress and metabolic pathways associated with cellular homeostasis.

In addition, IBD is clearly associated with well-defined genetic syndromes like Turner´s syndrome and Hermansky-Pudlak syndrome. Although genetic alteration in any one of several individual genes in mice is sufficient to lead to an IBD-like syndrome, genome-wide association studies (GWAS) have implicated over 160 distinct susceptibility loci for IBD.

Nucleotide-binding oligomerization domain-containing protein 2 (NOD2, also called IBD1 or CARD15), the first confirmed IBD gene, whose mutations confer increased risk of CD [251]. NOD2/CARD15 is expressed in intestinal epithelial cells (including, notably, Paneth cells) and mononuclear cells, and likely serves as a key component of the innate mucosal response to luminal bacteria [252].

Variants of the IL-23 receptor (IL23R) gene were found in both CD and UC [253], being in this case protective [254]. IL-10R signaling components have also been implicated, including IL10RA polymorphisms, streptothricin-acetyltransferase gene (SAT3), tyrosine kinase 2 gene (TYK2), Janus kinase 2 (JAK2) and interleukin 10 (IL-10) itself.

An unsuspected role for autophagy in IBD was recently described, implicating two component genes, autophagy related 16-like 1 gene (ATG16L1) and immunity-related GTPase family M gene (IRGM) [255]. Similarly, genetic variants that perturb mechanisms that protect against endoplasmic reticulum stress can signal apoptosis and can affect intestinal homeostasis in IBD [256]. In addition to coding variants, non-coding single nucleotide polymorphisms (SNPs) have shown to confer susceptibility to CD [257]. Furthermore, genetic changes may affect transcription-factor-binging sequences, locus accessibility,

translational efficiency and trans-regulators such as non-coding RNAs and microRNAs (miRNAs). In this regard, IBD-implicated loci contain more than 10 miRNA-encoding sequences and 39 large intervening non-coding RNAs (lincRNAs), supporting the notion that regulation of gene expression by miRNAs and lincRNAs may be mechanistically relevant in IBD [254].

4.4.2. Environment influences

Some environmental factors such as smoking, diet, infections, obesity, nursing, and various medications have been established firmly as influences on the development of IBD, but there are conflicting data and the precise impact in so many cases remains unclear.

- Smoking: Cigarette smoking is probably the most extensively studied and reported exposure associated with IBD. Cigarette smoking increases the risk and recurrence for CD and may be protective of the development of UC [258-261]. The risk of UC increases two years after cessation of smoking and persists for over 20 years. The increase in risk associated with smoking cessation may be explained by loss of the protective effect of smoking, which then precipitates the onset of or unmasks the symptoms of UC.
- Diet: Given the constant intestinal exposure to numerous luminal dietary antigens, it seems reasonable to postulate a relationship between diet and IBD. However, specific pathogenic antigens have not been identified. Much of the confusion may stem from the difficulty in reconstructing the relevant diet. While studies attempting to associate specific diets with the development of IBD have had inconsistent results, the data suggest that a 'Western' style diet (processed, fried, and sugary foods) is associated with an increased risk of developing CD, and possibly UC. A number of studies have implicated dietary risk factors with IBD, such the hypersensitivity to cow's milk protein in infancy [262], refined sugar intake [263-265], decreased vegetable and fiber intake [264, 266], increased dietary intake of total fat, animal fat, polyunsaturated fatty acids, and milk protein [265-268]. In addition, several reports have supported the treatment of IBD with fish oil preparations containing n-3 polyunsaturated fatty acids (PUFAs) [269, 270]. The dietary intake of n-3 PUFAs may modulate immune responses by decreasing the production of cytokines and leukotriene B_4, an inflammatory mediator with potent neutrophil chemotactic properties [271]. Interestingly, the incidence rates of IBD are lower in Japan, a country with high fish consumption [266, 272].

- Obesity: It is unclear if obesity is associated with an increased risk of developing IBD [273, 274]. However, accumulation of intra-abdominal fat may contribute to mucosal inflammation thereby affecting the clinical course in patients with established IBD [275-277].

- Infections: Dysbiosis or an imbalance in the gut microbiome may contribute to the development of IBD. It is suggested by the correlation between specific microorganisms and acute gastroenteritis and IBD [278-281]. As an example, an association between CD susceptibility and specific infectious agents (eg, measles virus, *Mycobacterium paratuberculosis*, paramyxovirus) has been suggested but remains unproven [282-286]. One prominent hypothesis is that normal intestinal microflora may contribute to the development of IBD in susceptible individuals. This is supported by the observation that animals that are genetically altered to be susceptible to IBD do not develop the disease when raised in a germ-free environment [287, 288].

- Nursing and other perinatal events: Breastfeeding stimulates the development and maturation of the gastrointestinal mucosa of infants and may protect them from gastrointestinal infections in infancy [289, 290]. There are studies that have not confirmed the association of breastfeeding with CD [291] or UC but did confirm the increased incidence of diarrheal illness during infancy in those who later developed UC [292, 293].

- Antibiotics: It has been hypothesized that antibiotic use by altering the gut flora may be a risk factor for IBD. While antibiotic use has been associated with IBD, it is unclear if this is a causal association [291, 294-296]. However, recent studies show how immunomodulatory antibiotics, especially minocycline, have been shown to represent an advantage in experimental models of IBD, when compared with traditional ones, due to their ability to restore the microbial balance in the intestine while controlling the deregulated immune response [297-299].

- Isotretinoin: An association between isotretinoin, used in the treatment of acne vulgaris, and IBD has been suggested by case reports [300-307].

- Nonsteroidal antiinflammatory drugs: as mentioned above, NSAIDs may increase the risk for the development of IBD with a variety of effects ranging from asymptomatic mucosal inflammation to strictures, obstruction, perforation, and major hemorrhage [308]. NSAIDs block the cyclooxygenase pathway of arachidonic acid metabolism [309]. Given the possible role of prostaglandins in intestinal

inflammation, it is not entirely surprising that these potent drugs might influence IBD. Available data pertaining to COX-2 selective inhibitors suggest that most patients can tolerate at least short-term treatment without exacerbation of disease [310, 311]. Cyclooxygenase-mediated disruption of the intestinal epithelial barrier associated with aspirin or NSAID use can affect the interaction between the gut microbiome and immune cells in the intestine lining. In addition, NSAIDs and aspirin alter platelet aggregation, the release of inflammatory mediators, and microvascular response to stress, which are key events in the pathogenesis of IBD. While a number of reports suggest that NSAIDs increase the risk for the development of IBD [312, 313] and may exacerbate underlying IBD [314-317], the absolute risk appears to be small and some patients with IBD appear to be able to tolerate NSAIDs, particularly when given in low doses [318, 319].

- Oral contraceptives and hormone replacement therapy: they may predispose to the development of IBD through thrombotic effects on the microvasculature. However, there are conflicting data concerning an association and, if present, the risk appears to be small [320-323].

- Appendectomy: A number of studies have suggested that appendectomy may protect against the development of UC, but the mechanism of the protective effect is unknown [281, 324-327]. One hypothesis is that alterations in mucosal immune responses leading to appendicitis or resulting from appendectomy may attenuate the pathogenetic mechanisms of UC [328, 329].

- Psychosocial factors: Studies examining the association between psychological factors and the risk of developing IBD have yielded inconsistent results [330-332]. However, stress may have a role in the exacerbation of symptoms in patients with established IBD, possibly via activation of the enteric nervous system and the elaboration of proinflammatory cytokines [333, 334].

4.4.3. Immunological and microbial factors

The physical barrier of the intestinal epithelium is complemented by a well evolved mucosal innate immune system, which is poised to defend against pathogenic incursions, and limit inflammatory responses to maintain a state of hyporesponsiveness to commensal bacteria. However, it is also one effector arm that mediates intestinal inflammation. Thus, the immune response has long been implicated in the pathogenesis of IBD. From a vast body of literature investigating the roles of both host and microbial factors, two basic themes in the pathogenesis of IBD emerge [335]:

(1) Dysregulation of the innate and adaptive immune system directed against luminal bacteria or their products found in the intestinal lumen.

(2) Inappropriate immune responses to organisms in the intestine that normally do not elicit a response, possibly due to intrinsic alterations in mucosal barrier function.

An exaggerated immune response against luminal components has been proposed to be the cause of IBD in genetically predisposed individuals, although many aspects of this response are not known accurately and/or globally to date. The activated immune response characteristic of IBD is dominated by mucosal CD4+ lymphocytes [4, 336]. Traditionally, in the context of the Th1/Th2 paradigm, it was established that CD differed from UC in that CD seemed to be a Th1 cytokine-mediated disease characterized by increased T cells producing interferon gamma (IFN γ) [337, 338] and IL-12 [339, 340], with activated STAT-4 [341]. By contrast, UC seemed to be a Th2 type-like cytokine profile, characterized by increased production of growth factor β (TGF-β) and IL-5, but without the altered expression of IL-4, the other prototypic Th2 cytokine [342].

After the identification and characterization of the new Th17 subset, the IL-17-producing Th17 cells in CD mucosa [343, 344], recent evidence suggests that alterations in Th1 and Th2 populations can actually result from the down-regulating effects of their products on this new Th17 cell population. In addition, the microbiota has an important role in the preferential localization of Th17 cells in the gut [345].

The cytokines associated with the Th1/Th17/Th2 spectrum are mainly responsible for the distinctive type of inflammation characterizing the form of IBD with which they are associated. There are, however, a well-known group of additional cytokines produced by epithelial, immune and smooth muscle cells, such as TNFα, IL-1β, and IL-6, that are more promiscuous in their function because they are associated with both forms of IBD to a lesser or greater degree [336]. In addition, reduced numbers of regulatory T cells (Treg) characterized by CD4+, CD25+ and Foxp3, which produce IL-10 and/or TGF-β, might be equally important [346]. The microbiota also has a role in promoting intestinal Treg-cell responses, since Treg-cell accumulation in the colon is reduced in germ-free mice and can be increased by particular indigenous bacteria [347].

Finally, there are other cellular types that can mediate the maintenance and development of IBD, like CD8+ T cells [348, 349], natural killer T (NKT) cells [350] and natural killer (NK) cells [351], dendritic cells [352, 353] and antibody producing cells or B lymphocytes [354], among others. Increased effector processes and a decrease in the function of regulatory T cells and various inflammatory cytokines may lead to imbalance and consequent immune chronic inflammation.

4.5. MEDICAL MANAGEMENT OF IBD

Medical treatment of IBD does not provide a cure for the disease. Thus, the main goal is to induce and maintain remission, to improve health related quality of life and to prevent disease complications. A variety of treatments exists, with different advantages and disadvantages. The current medical treatment of IBD relies upon the use of anti-inflammatory and immunosuppressive drugs with limited specificity, severe side effects and limited long term benefits [355, 356].

- Aminosalicylates: 5-aminosalicylic acid (5-ASA), also called mesalazine, has been shown to exert anti-inflammatory effects in IBD, especially in UC, along with 5-ASA derivates [357, 358]. Aminosalicylates have been used for a long time, as is the case with sulfasalazine (5-ASA linked to sulfapyridine), the first drug to induce remission in active UC [359]. Aminosalicylates are well tolerated with moderate adverse effects [360].
- Glucocorticoids (GCS): GCS are efficacious for the treatment of active IBD and are often added to induce remission [361, 362], but not as a maintenance therapy [363]. But GCS treatment is also associated with well-known side effects, such as weight-gain, hyperglycemia and diabetes, acne,

cutaneous striae, cataract, osteoporosis and mood disorders [364, 365]. Budesonide, a second generation GCS, has fewer side effects and enhanced anti-inflammatory activity is a standard drug for ileocolonic CD [365, 366]. These drugs may have resistance and create dependence, which limits their use in clinical practice.

- Immunomodulators: 6-mercaptopurine, azathioprine [367, 368], tacrolimus, cyclosporine [369] or methotrexate, are options if intravenous steroids fail for maintenance of remission [361, 370]. Immunomodulators may provoke allergy and serious toxicity. Some of them are also used increasingly for maintenance of remission.
- Anti-TNF: Infliximab, a mouse-human chimeric monoclonal tumor necrosis factor alpha antibody, is useful for induction of remission and maintenance treatment of therapy refractory IBD [371, 372]. However, there is a risk of severe opportunistic infections including tuberculosis, which may limit extensive use of TNFα antibody treatment [373]. Other immunomodulators are: adalimumab, certolizumab, etanercept. Adalimumab was constructed from a fully human monoclonal antibody and etanercept is a TNF receptor-IgG fusion protein. Certolizumab pegol is a monoclonal antibody derivative conjugated with high molecular weight poliethyleneglycol, directed against tumor necrosis factor alpha. There are also concerns about cost and long term adverse effects. It is also important to mention others TNF implicated drugs, like small molecular size TNF inhibitors.
- Other categories of treatment modalities include antibiotics or probiotics.

In spite of the wide range of drugs used in the treatment of IBD, therapeutic options currently available are not entirely satisfactory. This is why surgery is still an important therapeutic alternative in IBD, as colectomy in UC and anastomosis in CD. Endoscopic balloon dilation is a safe and effective alternative to intestinal resection in some cases [374].

Therefore, studies focusing on the pathophysiology of the disease that highlight new therapeutic targets are crucially important. In the last decades more and more animal studies have been published concerning the role of ion transport mechanisms in intestinal epithelial cells (colonic crypts) in the pathogenesis of IBD [375-377].

5. EPITHELIAL DISRUPTION IN IBD

Epithelial cells (and the overlying mucus) are the first barrier along the gastrointestinal tract preventing luminal microorganisms, especially infectious pathogens, from entering the body. Alterations in this barrier may be important factors in the pathogenesis of IBD. Although still inconclusive, alterations in intestinal mucus and high numbers of bacteria within the mucus have been associated with IBD [378-380]. Similarly, epithelial integrity may be compromised in inflammatory conditions, owing to the presence of noxious luminal factors (e.g. enterotoxins) and damage by inflammatory mediators. Epithelial cell apoptosis may be also a contributing factor [381]. The occurrence of discontinuities in this barrier may conceivably favor fluid leakage to the lumen from the *lamina propria*, contributing to diarrhea. On the other hand, increased leakiness is considered to facilitate the mucosal penetration of luminal antigens and/or microorganisms, thereby making the mucosa more vulnerable to inflammation, at the same time that increased permeability may exacerbate immune system activation. Augmented permeability has been documented in first-degree relatives of IBD patients without other signs of disease [380]. One of the main IBD models is based on this concept, namely the disruption of the epithelial layer by dextran sodium sulfate (DSS), suggesting that this is a relevant mechanism *in vivo*. Permeability changes in IBD appear to be related to augmented TJ permeability, including increased susceptibility to noxious factors in uninflamed areas [381-383]. Increased transcellular ion transport has also been reported [384]. Interestingly, barrier defects may be reproduced in intestinal cell lines with inflammatory mediators, namely IFNγ, TNFα, IL-1β or IL-13 [385-387], while IL-4 and IL-10 may have opposite (protective) effects [388, 389] .

It should be noted that these disturbances may lead to gross epithelial leaks, so epithelial cells may be themselves damaged by the inflammatory reaction. Indeed, the ulcerated condition of the mucosa gives its name to one of the two main manifestations of IBD, i.e. UC. The epithelium has a great capacity to seal off permeability defects. Even in case of mucosal necrosis, which occurs in the early stages of the TNBS model of colitis, the increase in conductance is only moderate [390, 391]. In fact, such an increase is no longer detected in chloride free conditions in this model (indicating that the conductance for other ions is not affected) [391]. In agreement with this observation, Cl^- replacement has been found to lower tissue conductance to a greater extent in inflamed human tissue than in the control [39]. Restitution, a prostaglandin/nitric oxide mucosal repair mechanism executed by cells adjoining epithelial lesions, appears to be critical in controlling excessive fluid leakage and antigen permeation [392, 393].

5.1. Inflammatory mediators: role of prostaglandins

Inflammation is amplified by a broad spectrum of inflammatory mediators such as eicosanoids, chemotaxins such as IL-8 and platelet-activating factor, IL-1β, TNFα [394], IFNγ or other chemotactic factors such as complement fragments (e.g., C5a). Inadequate production of proresolving mediators or the inability of these mediators to execute their antiinflammatory effects may exacerbate an inflammatory disorder and could represent an important stage in the progression from acute to chronic inflammation.

Eicosanoids [395] are a group of 20-carbon fatty acids and peptide-fatty acids derived from membrane phospholipids. In mammals, the principal precursor for eicosanoid synthesis is arachidonic acid, which is cleaved from membrane phospholipids via the action of phospholipases, mainly phospholipase A_2 (*Illustration 11*).

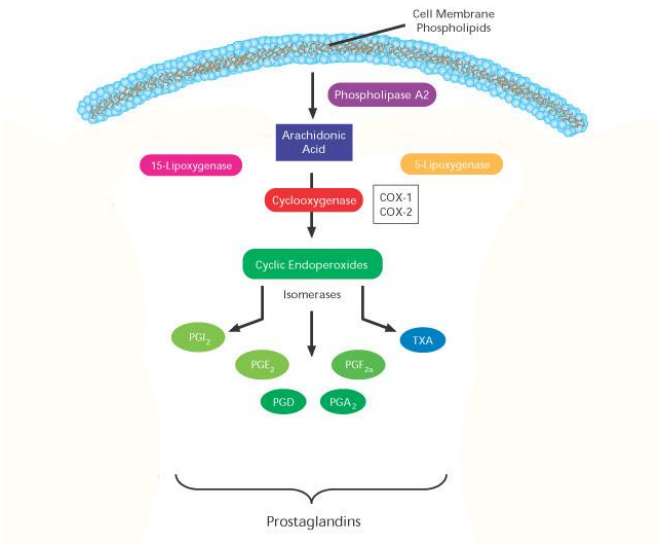


Illustration 11. *Eicosanoids cascade and receptors*.

Cyclooxygenase enzymes (COX-1 which is constitutive and COX-2 which is mostly inducible) reduce arachidonic acid to PGH_2, which is converted to five primary active metabolites, prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostacyclin (PGI_2), and thromboxane (TX) A_2 by the action of specific synthases (PGD synthases, PGE synthase, prostacyclin synthase and thromboxane synthase) [396-398], whereas the synthesis of leukotrienes (LT), lipoxins and another derivates is dependent on the actions of different lipoxygenases (5-,12-,15-LOX) [399].

All these eicosanoids exert their actions through a family of GPCRs (*Illustration 11*): prostaglandin D receptor (DP), EP_1, EP_2, EP_3 and EP_4 subtypes of prostaglandin E receptor,

prostaglandin F receptor (FP), prostaglandin I receptor (IP) and thromboxane A receptor (TP), and one GPCR in a different family, $CRTH_2/DP_2$ [400].

By and large, eicosanoids are thought of as proinflammatory mediators: prostaglandins have actions on vascular smooth muscle, neutrophil secretion, adherence and motility, and gastric acid secretion. However, this may be oversimplistic because PGs can exert potent antiinflammatory effects under certain conditions. Thus, PGs also have been shown to exert protective effects in the stomach, small intestine, and colon, although the mechanism of action remains unclear [401-404]. This PG immunomodulatory effect has been demonstrated also in IBD. Through mechanisms that are not fully understood, exogenous PGs have been shown to reduce the severity of experimental colitis [405-411]. Although this effect may be to some extent attributable to the effects on neutrophil function, PGs also seem to increase the resistance of the epithelium to injury. Additional evidence for a role of PGs as antiinflammatory factors in the intestine is the demonstration in experimental models that inhibitors of PG synthesis exacerbate the intestinal injury [412, 413] as well as numerous clinical reports of exacerbation of IBD by NSAID, which inhibit COX [314]. Thus, PGs have regulatory roles in normal physiological as well as pathological contexts.

5.1.1. PGD_2: dichotomous nature and action

LOCALIZATION	PGD_2	H-PGDS	L-PGDS	DP_1 (DP)	DP_2 (CRTH2)
Osteoblasts	•				
Megakaryoblasts		•			
Platelets	•				
Leukocytes (basophils, eosinophils, monocytes)				••	••
Th1 cells				•	•
Th2 cells	•	•		•	••
Mast cells	•	•			••
Macrophages	•	•	•		
Dendritic cells	•	•		••	•
Tissue			Brain, heart, kidney, lung	Small intestine retina, ileum, lung, stomach, uterus	Brain, heart, intestine, thymus, spleen, liver, lung, liver, kidney, brain, heart, thymus, spleen

Table*3.* *Localization of PGD2, H- and L- PGDS synthases and DP_1/DP_2 receptors. Black color: human. Orange color: mouse.*

PGD_2, an acidic lipid mediator, is the main COX metabolite generated by mast cells that are activated following allergen exposure by its crosslinking with the high-affinity receptor for immunoglobulin E [414, 415]. Other cell types that can synthesize small amounts of PGD_2 include platelets, alveolar macrophages, Th2 cells, dendritic cells (DCs) and osteoblasts [414-416] (*Table 3*).

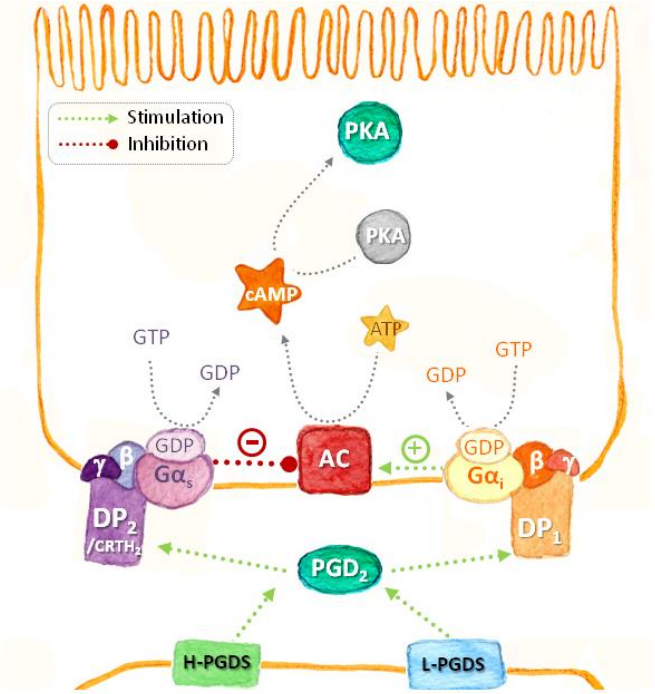


Illustration 12. *PGD_2 synthesis (H-PGDS & L-PGDS) and receptors (DP_1 & DP_2). Positive and negative regulatory influences are shown in green and red respectively.*

The synthesis of PGD_2 from its precursor PGH_2 is catalyzed by two PGD synthases (PGDS): H- and L-PGDS (*Illustration 12: PGD_2*). PGD_2 is further converted to 9α, 11β-PGF_2, a stereoisomer of $PGF_{2\alpha}$, which exerts various pharmacological actions different from those induced by $PGF_{2\alpha}$. PGD_2 is also readily dehydrated to produce prostaglandins of the J series, such as PGJ_2, Δ12-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15-PGJ_2) [417, 418]. This last product has been identified as an anti-inflammatory lipid acting as an endogenous ligand for the nuclear receptor peroxisome proliferator-activated receptor γ [419]. Therefore, the net effect may depend on the rate of production of distal products of PGD_2 depending upon the disease process.

5.1.2. PGD synthases: H-PGDS and L-PGDS

As mentioned above, two PGD synthases have been identified: H-PGDS (hematopoietic) and L-PGDS (lipocalin type) [420-423]. Both enzymes may form PGD_2 *in vitro,* but it is not fully understood which PGDS enzyme predominates under varied conditions *in vivo*. Both differ biochemically in terms of their amino acid sequence, tertiary structure, evolutional origin, chromosomal and cellular localization, and tissue distribution, and functionally, in terms of their biological relevance [421, 424].

- Hematopoietic PGD Synthase

H-PGDS, previously known as the spleen-type PGDS [420, 425], is a homodimer localized in antigen-presenting cells and mast cells of a variety of tissues and is involved in the activation and differentiation of mast cells. It is also expressed in dendritic cells, Th2 cells, Langerhans cells (dendritic cells of the skin and mucosa), and megakaryoblasts [426] (*Table 3*). While H-PGDS is proinflammatory in allergic airway diseases, it has shown to be protective in other models of inflammation [427, 428]. Thus, H-PGDS may act as an internal braking signal essential for bringing about the resolution of Th1-driven delayed type hypersensitivity reactions [429, 430]. In fact, inhibitors of H-PGDS have been shown to be protective in mouse models of allergic airway inflammation [431]. The compound HQL-79, a specific inhibitor of human H-PGDS, exhibits a therapeutic effect when used in animal models of allergic disease and neuroinflammation [432]. Thus, selective inhibitors of H-PGDS may prove to be more useful to suppress allergic and inflammatory reactions than COX-1 or COX-2 inhibitors, such as aspirin, indomethacin, and coxibs, because these COX inhibitors suppress the production of all PGs in comparison to H-PGDS inhibitors [433-435].

- **Lipocalin-Type PGD Synthase**

L-PGDS is a bifunctional protein, acting as a PGD_2-producing enzyme as well as an intercellular transporter of retinoids or other lipophilic molecules [436]. It is identical to beta trace protein, which was discovered in 1961 as a major protein of human cerebrospinal fluid [26–28]. Because it resembles lipophilic ligand carrier proteins it was named lipocalin-type PGD synthase. Structurally it is a monomer with a *β*-barrel structure and a hydrophobic pocket. It is present in the brain [421, 432, 437], heart, kidneys [438, 439] and lungs [440, 441] (*Table 3*). The L-PGDS concentration in human serum fluctuates with circadian rhythmicity. A large body of evidence demonstrates that L-PGDS has several key regulatory roles that extend beyond its function in the brain [438, 439, 442, 443].

Similar to H-PGDS in models of allergic inflammation, L-PGDS has been shown to be proinflammatory. Fujitani et al. reported that L-PGDS transgenic mice exhibit strong allergic lung responses and eosinophilia [444] with enhanced allergic airway inflammation. In a model of chronic allergic dermatitis, the block of L-PGDS with an inhibitor led to significant attenuation of inflammatory response [445], which was also confirmed in CRTH2 knockout mice. The proinflammatory role of L-PGDS has also been suggested in human

ulcerative colitis. Hokari et al. showed that the level of L-PGDS mRNA expression is increased in UC patients in parallel with disease activity [446, 447]. However, the immunomodulatory effects of L-PGDS are less well studied, but it has been suggested an important role of L-PGDS in immunomodulation [441].

5.1.3. PGD_2 receptors: DP_1 *vs.* DP_2

The effects of PGD_2 are mediated through the activation of two seven-transmembrane GPCRs, DP_1, the D-prostanoid receptor 1, and DP_2, also known as chemoattractant receptor homologous-molecule expressed on T-helper-type-2 cells (CRTH2), which are linked to different signaling pathways (*Illustration 13*) [448]. Several cells of the immune system express both receptors.

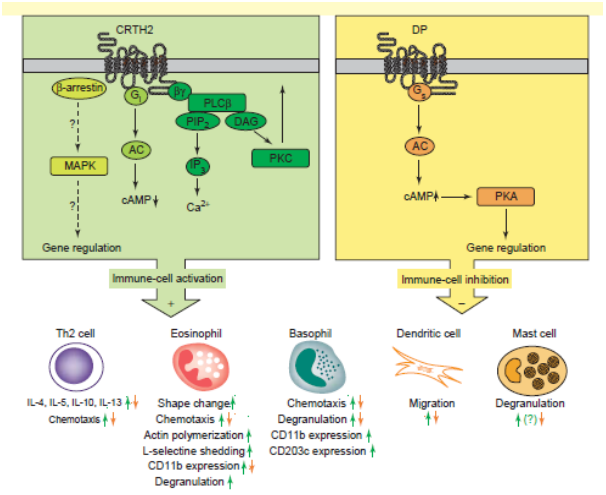


Illustration 13. *DP1 vs DP2 receptors. Figure (taken from Magney et al., 1986).*

- **DP_1 Receptor**

DP_1, also called DP receptor, the first identified receptor for PGD_2, has 73% homology at the amino acid level between the human and mouse forms [449]. DP_1 preferentially binds the ligand PGD_2, with a K_d of 1.5 nM, and binds with 100-fold or less affinity to PGE_2, $PGF_{2\alpha}$, iloprost and the TXA_2. Binding DP_1 by PGD_2 stimulates AC through $G_{s\alpha}$ proteins, resulting in increased intracellular cAMP levels and Ca^{2+} mobilization, but not the production of IP_3 (*Illustration 13*) [450]. Also PGD_2 induces the expression of Nrf2 in a DP_1 receptor-dependent manner [63]. In organ tissues, DP_1 is expressed at low levels in the small intestine and retina in humans [450], while in mice, DP_1 is found in the ileum, lung, stomach and uterus [451] (*Table 3*). In humans, DP_1 is expressed in basophils, eosinophils [452], monocytes, dendritic cells [453], and Th1 and Th2 cells [454] at low levels. In mice, monocytes, dendritic cells [453], and eosinophils [455] express DP_1 (*Table 3*). The increase of this secondary messenger inhibits platelet aggregation and causes bronchodilation and vasodilation in humans [414, 456]. DP_1 is

involved in the modulation of both innate and adaptive immune responses [457]. Thus, cell migration and/or activation can be inhibited in Jurkat cells, eosinophils, basophils, DCs and fibroblasts upon DP_1 transfection [452, 458-461], suggesting that DP might have an anti-inflammatory role in cells of the immune system. PGD_2 preferentially binds to DP_1 in spite of DP_2 [462], and its activation is largely thought to be responsible for the antiinflammatory effects of PGD_2.

- **DP_2 Receptor**

CRTH2, also known as Gpr44 G protein-coupled receptor 44, discovered in 1999 by Nagata et al. [463] and now known as DP_2, differs from DP_1 in its biological functions and cellular distribution [414, 415, 456, 459]. In contrast with DP_1, activation of DP_2 inhibits AC through $G_{i\alpha}$ proteins (*Illustration 13*), causing a decline in intracellular cAMP levels, intracellular Ca^{2+} mobilization, and generation of DAG and IP_3. Recent evidence suggests that DP_2 signaling might occur in the absence of PGD_2 production and independently of PGD synthase [417, 418]. Interestingly, the tissue expression pattern of DP_2 in humans differs from that in rodents [414, 415, 464]; Thus, DP_2 mRNA is expressed in various human tissues, including brain, heart, intestine, thymus, spleen, liver, and small and large intestine [465], while in the mouse, DP_2 mRNA is found at low levels in the lung, liver, kidney, brain, heart, thymus, and spleen [466] (*Table 3*). In human peripheral blood leukocytes, DP_2 is found on Th2 cells, basophils, eosinophils, and in low quantities on monocytes, but not on Th1 cells, CD 19+ B cells, NK cells, immature dendritic cells, or neutrophils [453, 466]. In sharp contrast, mouse DP_2 is expressed on both Th1 and Th2 cellular subsets {Abe, 1999 #605;Spik, 2005 #600}, in addition to eosinophils and monocytes, mast cell, and B cell lines [455] (*Table 3*). These differences suggest that DP_2 function might differ in humans and rodents. So far, DP_2-mediated signals *in vitro* and *in vivo* have been predominantly categorized as proinflammatory. Thus, DP_2 amplifies Th2 cell responses by inducing their migration, enhancing their adhesiveness to endothelial surfaces by increasing CD11b surface expression, and invoking further elaboration of Th2 cytokines [417, 418, 452, 455, 459, 467, 468].

On a cellular level, DP_1 and DP_2 can be regarded as antagonic receptors with opposing signaling pathways: DP_1 increases intracellular cAMP levels and limits DP_2 activation upon exposure to PGD_2 [468] (*Illustration 13*), while DP_2 decreases cAMP levels and mediates pro-inflammatory and pro-stimulatory effects [468]. It is thus tempting to hypothesize that these two PGD_2 receptors exhibit opposite functions in cells.

The physiological functions of PGD_2 include regulation of sleep and body temperature, olfactory function, intraocular pressure, hormone release, and nociception in the central nervous system. As noted, PGD_2 also prevents platelet aggregation and induces vasodilation and bronchoconstriction [414, 456]. In allergic diseases it is responsible for the symptoms in mastocytosis patients, such as flushing, diarrhea, tachycardia, dyspnea, and deep sleep [469].

PGD_2 may exert immunomodulatory effects. Thus, PGD_2 has been implicated in the initiation and progression of inflammation because it stimulates the chemotaxis of Th2 cells, eosinophils and basophils both *in vitro* and *in vivo* [452, 459, 460, 470], and it also evokes long-term alterations in colonocyte and barrier function [471-473]. But, on the other hand, PGD_2 has antiinflammatory properties, with functions in resolution of inflammation [396, 474]. For instance, in a model of experimental colitis COX-2-derived PGD_2, acting via the DP receptor, was shown to attenuate neutrophilic infiltration into the colonic mucosa [471, 475]. In addition, increased PGD_2 levels were shown to be protective in patients with IBD by regulating mucosal blood flow [411]. This information strongly supports the anti-inflammatory effects of PGD_2 [396, 429, 471, 473].

PGD_2 EFFECT AND EXPERIMENTAL OBSERVATION	EFFECT	MOLECULAR TARGET	REFS
Overexpression of H-PGDS in mice enhaces adipogenesis and increases insulin sensitivity caused by overproduction of PGD_2	Pro-inflammatory	Not known	
$DP^{-/-}$ mice have reduced asthmatic responses through reduced PGD_2	Pro-inflammatory	DP_1	
PGD_2 from H-PGDS induces neuroinflammation in Krabbe´s disease	Pro-Inflammatory	DP_1	
PGD2 is involved in the initiation and maintenance of the allergy in mice	Pro-Inflammatory	DP_1 and DP_2	
Overexpression of L-PGDS in mice results in enhanced pulmonary inflammation that follows allergen challenge	Pro-Inflammatory	Not known	
inhalation of aerosolized PGD2 enhances eosinophilic and lymphocytic airway inflammation in mice upon allergen exposure	Pro-Inflammatory	Not known	
PGD2 induces expression of the gene encoding COX-2 via activation of NF-κB	Pro-Inflammatory	Not known	
PGD2 enhances leukotriene-C4 synthesis by eosinophils during allergic inflammation *in vivo*	Pro-Inflammatory	Not known	
PGD2 stimulates chemotaxis of eosinophils in vitro	Pro-Inflammatory	DP_2	
PGD2 stimulates chemotaxis of eosinophils in vivo	Pro-Inflammatory	DP_2	
PGD_2 stimulates chemotaxis of Th2 cells in vitro	Pro-Inflammatory	DP_2	
PGD_2 stimulates chemotaxis of basophils in vitro	Pro-Inflammatory	DP_2	
PGD_2 antiinflammatoy in a mouse model of pleuritis	Anti-Inflammatory	Not known	
PGD_2 antiinflammatoy in a mouse model of colitis downregulating granulocyte infiltration	Anti-Inflammatory	DP_1	
PGD_2 is neuroprotective by inducing astrocytes to produce neurotrophins	Anti-Inflammatory	Not known	
PGD_2 is neuroprotective in conditions of neuronal injury	Anti-Inflammatory	DP_1	
PGD_2 regulates mucosal blood flow in patients with IBD	Anti-Inflammatory	DP_1	
PGD_2 inhibits activation of eosinophils, basophils, dendritic cells and fibroblasts	Anti-Inflammatory	DP_1	

Table 4. *PGD_2 effects and experimental observations.*

Thus, considering all of the above, the double dual nature of the two synthases and the two receptors, plus the different metabolite derivates from this prostanoid, suggest that PGD_2 is a key mediator in inflammatory diseases, because it can promote and inhibit inflammatory processes depending on

the animal species, the organ and the inflammatory stimulus. A summary of this dual effect of PGD_2 is shown in the *Table 4*.

5.2. Disturbances in ion secretion

Because diarrhea is a hallmark of intestinal inflammation, particularly colitis, increased ionic secretion was initially suspected as the main causative factor. In fact, most inflammatory mediators have secretory effects *in vitro*, including PGs [6], leukotrienes [7, 8], platelet activating factor [9], histamine [10], IL-1β [12, 13], TNFα [14, 15], reactive oxygen and nitrogen metabolites [16, 476], and even mucosal supernatants obtained from IBD patients [17]. This is why for many years it was assumed that diarrhea was the logical consequence of the relative abundance of inflammatory mediators brought about by mucosal inflammation. However, examination of transport in actually inflamed intestinal tissue revealed a completely different picture. Colonic short-circuit current (Isc) in Ussing chamber mounted tissue, which largely reflects chloride/bicarbonate secretion, is actually diminished in the basal state (i.e. in the absence of exogenously added secretagogues) compared to noninflamed specimens [18-23, 25, 26, 390, 477-480]. Furthermore, when inflammatory mediators were used to stimulate secretion (in inflamed tissue), the change in Isc was also lower than that observed in controls. This finding was observed consistently both in animal models and in humans, and extends also to some other pathological conditions including irradiation [481-483], parasitic infestation [484-486], bacterial infection [487], oral endotoxin [488], IL-1β/IL-6 treatment [489] and stress [490]. It should be noted however that this may not be the case for acute inflammatory episodes [491-495] and collagenous colitis [496]. Therefore diarrhea cannot be ascribed to excessive ionic secretion. Instead, the defect in secretion would be expected to limit diarrhea or to produce frank fecal dehydration and constipation. A decrease in ionic secretion, especially in response to inflammatory mediators, may be viewed theoretically as protective to the host, impeding excessive fluid and electrolyte loss in case of protracted inflammation. It is interesting to note that abnormalities in ionic secretion appear to linger even weeks after the inflammatory reaction has subsided [18, 24, 497].

Once the hyposecretory and hypoabsorptive status of the inflamed intestine have been established, the next question is to address why this happens.

Of course, the alterations in enterocytes just described are expected to result in changes in ionic transport that are difficult to reverse in the short term. However, a few studies have reported that some of these disturbances, namely inhibited secretion, can be at least partly revoked *in vitro* in animal models of

IBD. Thus, there are different regulatory mechanistic indications came from pharmacological studies that involve NO, cholinergic regulation, cAMP, enteric neural signaling and cytokines in this revoked of the disturbances in ion secretion (*Illustration 14*). These findings, added to the disturbances in the expression levels of the transporters, may explain the enterocyte transport situation in IBD.

5.2.1. NO and cholinergic regulation: The first mechanistic indication that can explain the downregulation of ion secretion in the inflamed intestine is NO. Actually, some pharmacological studies suggest its implication in chronic diarrhea associated with mild inflammation [31] as well as in collagenous colitis [32]. In addition, the iNOS selective inhibitor L-NIL (L-N6-(1-iminoethyl)lysine) has been shown to reverse the reduced secretory response to IBMX (3-isobutylmethylxanthine) and electrical field stimulation, although not to carbachol (an agonist of muscarinic and nicotinic receptors), in experimental colitis [18, 28]. These studies did not address the mechanism of NO inhibition. In an *in vitro* model using conditioned media from activated peripheral blood mononuclear cells to modulate T84 ionic transport, the McKay group reported that NO donors could prevent epithelial secretion by acting on the leukocytes but not on the epithelial cells [498].

On the other hand, later investigations suggested that NO may interfere with cAMP regulated secretion, including CFTR trafficking, by inhibiting AC in a guanylate cyclase independent fashion [30]. Thus nitric NO exerts inhibitory effects on intestinal secretion acting by direct and indirect mechanisms. However, it is unclear why cholinergic evoked secretion is not modulated by NO. The McKay group obtained additional interesting data relative to cholinergic regulation of ionic secretion in the inflammatory state. Using the DSS mouse model of colitis, they found reduced Isc responses to electrical nerve stimulation, forskolin and carbachol, as expected [499]. However, upon discontinuation of DSS, carbachol elicited a negative instead of a positive Isc response, which was linked to an action on enteric neurons dependent on nicotinic cholinergic receptors. This effect is unmasked by the lack of muscarinic cholinergic effect as a consequence of inflammation {Hirota, 2006 #656;Green, 2004 #270;Sayer, 2002 #271}. However, it is unclear why this reversal of carbachol effect is seen only during the recovery period. It was later found that the reversal of Isc is in fact dependent on an intact myenteric plexus [500]. Unlike the effect of carbachol described above [18, 28], NO does seem to be involved in this peculiar response. Specifically, the source of NO was identified as enteric glial cells {Green, 2004 #270}. An additional problem in the interpretation of these studies is that, as is often the case with this mediator, NO has an equivocal role in the regulation of

ionic transport as such, since there are conflicting reports by several groups. NO can elicit secretion directly in Caco-2 cells [33] and in the human colon [501]. In fact, NO has been involved in increased basal and stimulated ionic secretion in response to acute exposure to enteroinvasive bacteria, an effect dependent on cAMP/cGMP modulation [495]. Furthermore, NO has been proposed to mediate the actions of laxatives [35]. The complex regulatory effects of NO may depend on its concentration, source, and pathophysiological status of the host [34].

5.2.2.cAMP: As discussed above, the modulation of this second messenger has been found as an important target in the regulation of ionic transport [134]. Several studies have focused on the role of cAMP in the regulation of intestinal epithelial cells. By using a pharmacological approach based on analysis of concentration response curves our group found that the data obtained using carbachol and IBMX as Ca^{2+} and cAMP dependent stimuli, respectively, were consistent with a low activation of cAMP operated chloride channels, i.e. CFTR, in the TNBS model [26]. Thus, both curves had substantially lower maximal Isc values and the IBMX, but not the carbachol curve, was shifted to the right (higher PDE inhibition is needed to produce similar amounts of cAMP in the face of reduced cAMP formation). cAMP levels were indeed found to be severely reduced in isolated epithelial cells, supporting our hypothesis. Other investigators have focused on the role of AMP kinase, an enzyme that is upregulated by metabolic and oxidative stress, using the IL-10 knockout model [480]. AMP kinase was activated in parallel to the inflammatory response and its pharmacological modulation was found to correlate with the defect in induced epithelial secretion.

5.2.3. Enteric neural signaling: an additional clue to the mechanism of reduced secretion came from *in vitro* experiments performed with inflamed tissue devoid of the submucosa in the TNBS model. Unexpectedly, removal of the submucosa before mounting the mucosal sheets in Ussing chambers also reverses at least partly the defects in ionic secretion [26]. These changes are executed in a matter of minutes and therefore suggest that the changes in transport must be, at least in part, of regulatory nature. Such complete reversal indicates that the machinery required for epithelial ionic secretion must be intact and that it is somehow inhibited by the submucosa, probably involving submucosal neurons. Previous data from our group additionally point to a complex inhibitory action of enteric neurons on cholinergic stimulated secretion, consistent with previous findings [123]. The alterations in enteric neural signaling seem to be involved also in the spreading of disturbances in ionic transport to sites distal to the inflammatory focus. Thus, our group found that the (uninflamed) proximal colon exhibited a lowered response to Ca^{2+} but not cAMP-dependent stimuli in the presence of distal colitis, an effect that was abolished by tetrodotoxin or lidocaine [479]. Similarly, O'Hara et al. [502] described a higher colonic basal Isc and reduced response to veratridine (which

activates voltage-operated Na^+ channels) associated with altered function of submucosal neurons in guinea pig ileitis. In another study, jejunal ionic absorption was found to be inhibited by inflammation of the large bowel in a tetrodotoxin-dependent fashion [503] .Other authors have reported similar distal involvement of intestinal muscle and neuronal function [504, 505].

5.2.4. **Tolerance**: The reduction in the secretory response to inflammatory mediators may also reflect adaptation to continued stimulation, i.e., tolerance. For instance, *in vitro* pretreatment of uninflamed colon, but not small intestine, with histamine reduces the secretory responses to anti-IgE to levels comparable to those of IBD patients [19]. This hypothesis is not consistent with the fact that decreased secretion is prolonged beyond resolution of the inflammatory bout, as established by laboratory markers {Asfaha, 1999 #242;Green, 2004 #270}. Another possibility is that autolimiting signals, such as inositol 3,4,5,6-tetrakisphosphate and EGF receptor transactivation, may be involved in attenuating the response to secretagogues [506, 507].

5.2.5. PGD_2: Zamuner et al. {Zamuner, 2003 #265} identified a distinct mechanism for these late alterations, namely, increased PGD_2, supposedly not present during the inflammatory response, suggesting that this late disturbance may be a separate phenomenon. It should be noted that in a different model of inflammation inhibition of COX-2, the main source of prostaglandin D2, failed to reverse ion transport abnormalities [508].

5.2.6. Cytokines and protein expression: The issue of the impact of intestinal inflammation on expression of ionic transporters has been addressed by several groups *(see table 5).* The Na^+/K^+ ATPase is at the core of all ionic transport by the enterocyte, as it generates the electrochemical gradient necessary for vectorial transport. One study found unchanged mRNA of Na^+/K^+ ATPase, but lower protein expression, suggesting that both transcriptional and post-transcriptional mechanisms may be operative in these conditions [38]. But this pump has been generally shown to be downregulated [21] or inhibited in cellular models under the influence of TNFα or IFNγ, in experimental colitis, and in human IBD patients (*Illustration 14*) [41, 164, 509, 510]. In an elegant study by the Kim Barrett's group using a human fetal small intestinal xenograft model, long term exposure to IFNγ was found to produce a decrease in the expression of NKCC1 and the α_1 subunit of the Na^+/K^+ ATPase, with lowered ionic secretion [37]. Transport abnormalities can be reproduced at least partially in *in vitro* models. In particular, IFNγ has been described to decrease ionic secretion in the T84 cell

line and to reduce Na^+/K^+ ATPase activity and expression (α subunit), as well as that of NKCC1. This effect may be mediated partly by the EGF receptor {Colgan, 1994 #623;Fish, 1999 #622;Sugi, 2001 #621;Uribe, 2002 #624}. Interestingly, IFNγ is increased in NHE3 knockout mice and is thought to play a role in physiological compensation in this model, counteracting diarrhea [511].There is some evidence that IFNγ downregulates cAMP but not Ca^{2+} depending secretion in the T84 epithelial cell line, possibly by decreasing AC expression [512]. As mentioned before, this cytokine also produces barrier defects. In cell models TNFα seems to be able to enhance IFNγ effects [513] but has also been reported to potentiate the carbachol secretory response [514], while IL-10 [515] and IL-4 [389] inhibit ion secretion and promote absorption.

About CFTR, there are contradictory results, ranging from downregulation to an increased expression. Thus, some groups demonstrate no changes in the expression of this channel in different studies [26, 37], while there are groups that observe upregulation [36, 516] or downregulation [517] of CFTR in *in vivo* as well as *in vitro*. In summary, Na^+/K^+ ATPase seems to be downregulated, while the available studies concerning CFTR show conflicting results. About KNCC1, there is comparatively little information on this essential part of Cl^- secretion, but it appears also to be unaffected or downregulated (*Illustration 14*).

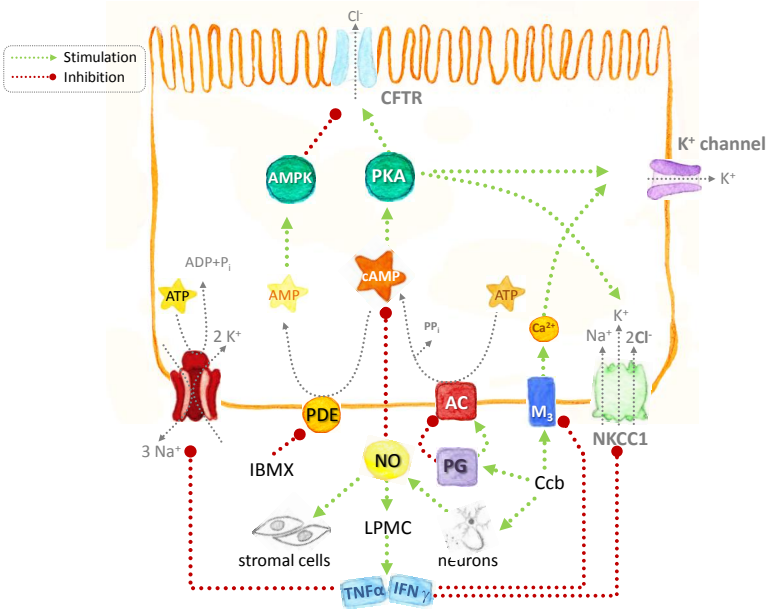


Illustration 14. *Regulation of intestinal epithelial ionic secretion in inflammation. Positive and negative regulatory influences are shown in blue and red, respectively. Different K channels are activated by Ca2 and cAMP signals. AC, adenylate cyclase; AMPK, AMP kinase; LPMC, lamina propria mononuclear cells; PDE, phosphodiesterase; PGR, prostaglandin receptor.*

Taken together, the evidence available suggests that reduced ionic secretion in the inflamed intestine involves nitric oxide and the enteric nervous system, operating in part on the epithelial cAMP signaling pathway reducing CFTR and NKCC1 activation, while inflammatory cytokines affect transporter expression preferentially (*Illustration 14*). These alterations appear to be at least partly reversible.

5.3. DISTURBANCES IN IONIC ABSORPTION

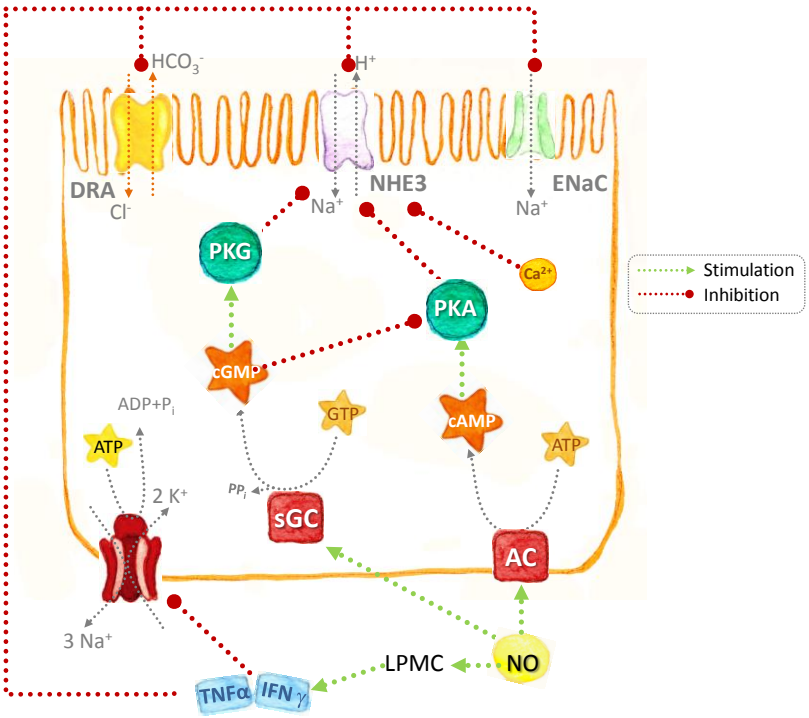


Illustration 15: *Regulation of intestinal epithelial ionic absorption in inflammation. Positive and negative regulatory influences are shown in blue and red, respectively. GC, guanylate cyclase; LPMC, lamina propria mononuclear cells; PKG, cGMP activated protein kinase.*

In addition to the potential contributing mechanisms from the reduced driving force for active transport, impaired expression, mislocalization, or defective transport function of Na^+ absorptive proteins have also been described (*Table 5*). Thus, Na^+ absorption has been long recognized to be significantly reduced in intestinal inflammatory states [21-23, 39, 496]. Actually, early studies from the 1970s had already demonstrated reduced Na^{22} absorption in colonic explants from affected patients [518, 519]. The mechanism of this phenomenon has received less attention than altered ionic secretion, possibly because measuring NaCl absorption is more technically cumbersome. But as is already described with

the disturbances in the hydroelectrolytic transport, there are different regulatory mechanistic indications coming from pharmacological studies that involve NO, cholinergic regulation, cAMP and cytokines are involved in the disturbances in ion absorption (*Illustration 15*).

5.3.1. NO and cholinergic regulation: As is already described with the downregulation of hydroelectrolytic transport, there are a few results that involve NO in the downregulation of ion absorption in the inflamed intestine. In addition to some pharmacological studies which suggest the NO implication in chronic diarrhea associated with mild inflammation [31] as well as in collagenous colitis [32], there is another study, in which jejunal ionic absorption was found to be inhibited by inflammation of the large bowel in a tetrodotoxin-dependent fashion [503]. Other authors have reported similar distal involvement of intestinal muscle and neuronal function [504, 505].

5.3.2. cAMP: As is shown above, the low epithelial cAMP levels are consistent with reduced secretion. However, since they could also lead arguably to enhanced absorption via NHE3 activation, it is likely that there are overriding signals at work.

5.3.3. Protein expression and cytokines: A number of reports exist on the expression and function of NHE3 in intestinal cells lines, where cytokine exposure results in a decrease in NHE3 mRNA expression, disappearance of NHE3 from the membrane, and, of course, decreased active Na^+ absorption (*Illustration 15*) [42, 520-522]. Also in chronic colitis animal models a downregulation in NHE expression has been described [42, 523]. Notably, NHE3-deficient mice spontaneously develop colitis restricted to the distal colonic mucosa, suggesting a key role of NHE3 in intestinal inflammation [375]. In addition, Lenzen H. et al. showed how in the IL-10 $^{-}/^{-}$ mouse colitis model there is downregulation of NHERF2 and PDZK1, in spite of unaltered NHE3 expression and localization, suggesting that these regulatory proteins may be an important factor leading to NHE3 dysfunction in IBD [524]. However, whether NHE3 expression or function is actually impaired in mucosa from IBD patients is not clear: there are studies reporting no significant decrease in NHE3 mRNA expression levels in UC patients [36, 525], studies reporting normal NHE3 immunostaining in UC mucosa [526], and a few studies reporting a decreased protein abundance for NHE3 as well as a number of other proteins in biopsy tissue from UC patients [164, 525]. Yeruva et al. showed how the transport capacity of NHE3 in the colonic mucosa from UC patients is defective, but with correct NHE3 location and abundance in the brush border [527]. Therefore, the molecular mechanism of NHE3 functional impairment remains unsolved, and it may be related to changes in the regulatory proteins that ensure NHE3 activity and

membrane retention [528]. The regulation of NHE1 on the basolateral membrane behaves opposite to the apical NHE3: Farkas et al. showed that the expression of NHE1 is strongly upregulated in UC, which is in accordance with elevation of functional activity [120]. NHE1 was shown to be upregulated in mouse colitis [376]. Interestingly, experiments in mice revealed that inhibition of NHE activity with amiloride strongly decreased the inflammatory processes *in vivo* in IBD mouse model [376]. Supporting this observation, NHE1 is rapidly activated in response to a variety of inflammatory signals, such as IL-1β [529], TNFα [530], IFNγ [531], and LPS [530]. Furthermore, functional NHE1 activity is required for both maximal NF-κB activation and IL-8 production in colonic epithelial cells [376]. The Cl^-/HCO_3^- exchanger DRA has been reported to be downregulated in UC and animal models of colitis based on mRNA analysis [36, 526]. In fact, in UC, Cl^- uptake by the colon is significantly reduced [532]. It has been described that there is a higher level of Cl^- in the colon of patients with UC compared to controls, suggesting that the normal luminal to mucosal exchange of Cl^- for bicarbonate is impaired [533]. However there are conflicting data at the protein level (*Table 5*) [36, 526, 534]. At least one group has found evidence of defective Cl^-/HCO_3^- exchange in experimental ileitis, limited to villus cells [535].

Gene	Change	References
Na/KATPase	↓ expression in IBD	[13, 15-18]
	↓ expression in ExpIBD (α1)	[19]
	↓ expression by IFN-γ	[20-25]
CFTR	↑ expression in UC	[26]
	= expression in ExpIBD	[10, 19, 20]
	↑ expression in IL13 transgenic mice	[27]
	↓ expression by IFN-γ	[22]
DRA	↓ expression in UC	[26, 28, 29]
	= expression in UC	[26]
	↓ expression in ExpIBD	[20, 29]
	↓ expression by IFN-γ	[30]
NHE3	↓ in UC (except in mild cases)	[26]
	↓ expression in IBD	[18]
	= expression in UC	[28, 31]
	↓ expression in ExpIBD	[18, 32]
	↓ expression by IFN-γ, TNF	[33, 34]
NKCC1	= expression in ExpIBD	[19]
	↓ expression by IFN-γ	[20]
ENaC	↓ expression in IBD	[13, 18, 25, 35, 36]
	↓ expression in ExpIBD	[32, 37]
	↓ expression (β/γ) by IFN-γ, TNF, IL-1β	[35, 36, 38]
NHE2	= expression in IBD	[18]
	↓ expression by TNF	[39]
NHE1	↑ in UC	[28]
	↓ expression in IBD	[18]
	↑ in ExpC	[18]
	↑ expression by IL-1, TNF, IFN-γ, LPS	[28]
NHERF1	↓ expression in IBD	[18]
	↓ expression in ExpIBD	[18]
CLC-5	↓ expression in IBD	[18, 40]
ASBT	↓ expression in IBD	[41]
	↓ expression in ExpIBD	[42]
ABCB1	↓ expression in UC	[43, 44]
BCRP	↓ expression in UC	[44, 45]
AQP	↓ expression AQP4, AQP8 in IBD and ExpIBD	[46]
OCTN1-2	↓ expression in IBD	[41, 47]
AC	↓ expression by IFN-γ	[48]

Table 5. *Changes in the transportome in intestinal inflammation at the expression level.*

On the other hand, ENaC expression is downregulated in IL-2 knock-out mice [21, 536, 537]. Furthermore, the response to aldosterone is severely impaired [523, 536]. In addition, UC is also associated with substantial decreases in the expression of the electrogenic ENaC β- and γ- subunits [38]. Interestingly, IFNγ is increased in NHE3 knock-out mice and is thought to play some role in physiological compensation in this model, counteracting diarrhea [511]. In cell models IL-10

[515] and IL-4 [389] inhibit ion secretion and promote absorption [538]. Using an *in vitro* model, it was found that proinflammatory cytokines (TNFα, IFNγ, IL-1β) decrease the expression of the β/γ subunits of ENaC, thus providing a mechanistic link with impairment of electrogenic Na^+ absorption [536, 539]. It has also been shown that IL-1β reduces DRA mRNA expression *in vitro* by inhibiting gene transcription.

Taken together, these studies suggest that NaCl absorption is inhibited in the inflamed intestine due to alterations in the expression, localization, defective function and regulation of the transporters involved, mainly NHE3, DRA, ENaC and the Na^+/K^+ ATPase. The role of signaling pathways other than cytokines is unclear (*Illustration 15*). Thus altered electrogenic Na^+ absorption may contribute significantly to diarrhea.

These regulatory findings in the hydroelectrolytic absorption and secretion, added to the disturbances in the expression of the different transporters, may explain the enterocyte transport situation in IBD.

NOTE: Previous to the experimental work included in this Thesis, we deeply studied the state of the art regarding the transepithelial ionic transport in the inflamed intestine. As a consequence, two review articles were published in international journals [43], [44].

AIMS

Inflammatory Bowel Disease (IBD) is a chronic relapsing inflammatory disorder of the gastrointestinal tract that encompasses two idiopathic and major inflammatory diseases: Crohn's disease and ulcerative colitis. Both forms of IBD significantly impair quality of life, and require prolonged medical and/or surgical interventions. What makes it particularly challenging is its still unknown cause, its unpredictable presentations and symptoms, the less than optimal treatments, and a rise in its incidence and prevalence in many areas of the world. Several nutraceutical agents may have intestinal antiinflammatory activity. The initial goal of this doctoral thesis was to assess the effect of several functional foods on intestinal ion transport in the colonic inflammation and to further characterize the associated pathophysiology of these alterations in IBD. These substances were studied were bovine glycomacropeptide and several oligosaccharides such as fructooligosaccharides, inulin, goat milk oligosaccharides and Active Hexose Correlated Compound. Unfortunately, against our expectations these agents showed a generalized lack of substantial modulation of ion transport function despite their well-established intestinal antiinflammatory activity in preclinical IBD models. Therefore we decided to move toward an in depth characterization of alterations related to the ionic transport of IBD itself.

The ionic transport situation in IBD is characterized by profound disturbances of both ion and water transport across the intestinal epithelium, which results in diarrhea and may contribute to the overall inflammatory response. The mechanisms underlying this phenomenom have been studied by us and other authors, but are still poorly delineated. Therefore the actual work of this doctoral thesis represents a continuation of previous efforts of our research group published a few years ago, along with those of other laboratories which have focused on the disturbances of ion transport in IBD. The scenario that can be drawn from this set of studies are summarized as follows:

- As opposed to acute colitis, which is typically accompanied by enhanced hydroelectrolytic secretion, chronic colitis in humans and animal models is characterized by inhibited ion and water absorption and secretion.
- These disturbances in ion transport have been linked in part to changes in the epithelial transportome, brought about by proinflammatory cytokines such as IFNγ or TNFα, but also by regulatory actions attributed to nitric oxide, PGD_2, changes in the cholinergic nervous system within the gut, or deficit in epithelial cAMP levels.

- Inhibition of secretion (Isc in Ussing chambers) is at least partly reversible at short term in vitro, suggesting that regulatory modulation of transport is involved in this phenomenon.

Based on all the above, we formulated 4 main objetives in this doctoral thesis:

1. To carry out an in depth characterization of the molecular basis of the ion transport defect in the inflamed intestine. Specifically, to assess whether changes in the abundance of proteins involved in vectorial ion transport (transportome) underlie the transport defect.
2. To elucidate the relationship between epithelial cAMP levels and downregulation of ion transport in IBD.
3. To identify the factors involved in the regulation of epithelial cAMP via Gi protein stimulation.
4. To validate, whenever possible, our findings in human samples and by the use of complementary technical approaches, in particular funcional genomics.
5. To carry out a technical assessment of Western blot analysis in relation to the requirement of the so called loading controls, applied to transporters and elsewhere.

MATERIALS & METHODS

1. EXPERIMENTAL SYSTEMS

Unless stated otherwise, all other reagents, including Trinitrobenzene sulphonic acid (TNBS), were obtained from *Sigma (Madrid, Spain)*.

1.1. CELL ISOLATION & CULTURE

- **Isolation of rat colonocytes**

Colonic epithelial cells were isolated from the distal colon of control and TNBS (non necrosed) rats (please see below: *TNBS model of rat colitis* for further details on the induction of colitis with TNBS) by calcium chelation and percoll purification. Briefly, the rats were sacrificed with overdose of isoflurane and the colon was obtained, gently flushed clean with ice-cold PBS (phosphate buffered saline), cut longitudinally and cleaned from the mesenteric adhesions and fat. The tissue was chopped and incubated in the *Medium I** for 20 min with shaking at 37 °C. By shaking the tube during incubation, epithelial cells were separated from the mucosa. Then, the sample was passed through a 70 mm cell strainer (*BD Bioscience*) into a 50 ml Falcon tube. After this, the part of the sample retained in the strainer was incubate again in *Medium I** with the same conditions, and passed through a 70 mm cell strainer. The two filtrated fractions were combined and centrifuged at 200 xg for 10 min at 4 °C. To finish, the pellet which contains colonocytes and intraepithelial lymphocytes (IEL) was resuspended in 8 ml of a 40 % (v/v) percoll solution, the resulting cell suspension was overlaid on top of a2 ml of the 70 % (v/v) Percoll solution and centrifuged at 200 xg during 30 min without brake. Colonocytes located in the white ring at the top of the percoll were carefully collected and washed with PBS. An aliquot from purified cell suspension was used to count cells in a hemocytometer under a phase contrast microscope following trypan-blue exclusion to distinguish the viable cell population.

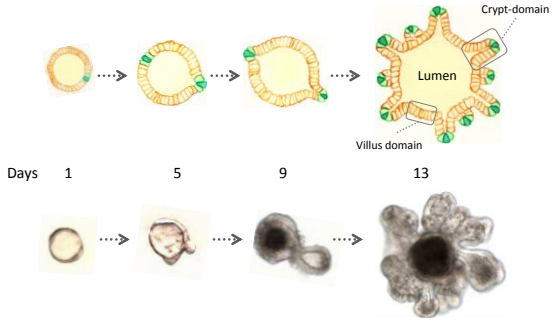
*Medium I** contains: HBSS (Hank´s balanced salt solution) with 2 % (v/v) of fetal bovine serum (FBS), 1 mM Dithiothreitol (DTT, reducing agent) and 1 mM ethylenediaminetetraacetic acid (EDTA, cation chelant).

- **Crypt isolation and organoid culture from mouse jejunum**

Mouse organoids were generated from isolated jejunum crypts and maintained in culture.

- **Crypt isolation:** Isolated jejunum from WT or Cftr$^{-/-}$ mice (CF mice) were opened longitudinally, and washed with cold PBS. The tissue was chopped into pieces of approximately 5mm, and further washed with cold PBS. These fragments were incubated in 2 mM EDTA with PBS for 30 min under slow rotation (40 g) at 4 °C. After removal of the EDTA medium, the tissue fragments were vigorously suspended using a 10 ml pipette with cold PBS. With this actively suspension the villi were separated from tissue fragments and floating in the supernatant. This supernatant was discarded, or saved for later RNA isolation; the sediment was resuspended with PBS. After further vigorous suspension and centrifugation (500xg 5 min), the supernatant was enriched for crypts. This fraction was passed through a 70 mm cell strainer (*BD Bioscience*) to remove residual villous material. Isolated crypts were centrifuged at 200 xg for 3 min to separate crypts from single cells. The final fraction consisted of essentially pure crypts and was used for culture or for the later RNA isolation.

- **Organoid culture** [540, 541]: A total of 500 crypts were mixed with 50 µl of Matrigel^{TM} (*BD Bioscience*) and plated in 24-well plates. After polymerization of Matrigel^{TM}, 500 µl of crypt culture medium (Advanced DMEM/F12 (*Invitrogen, Barcelona, Spain*)) containing growth factors (10–50 ng/ml EGF (*Peprotech*), 500 ng/ml R-spondin 1 (Rspo1)-conditioned medium (stably transfected Rspo-1 HEK293T cells were kindly provided by C.J. Kuo, Department of Medicine, Stanford, CA) and 100 ng/ml noggin (*Peprotech*)) was added.



Illustrtaion 16 & picture 3*. Establishement of intestinal jejunum organoids.* ***Illustration 16.*** *Schematic representation of a jejunum intesitnal organoid with the lumen corresponding to the intestinal lumen.*
Picture 3*. Time course of an isolated single cryot*

Growth factors were added every other day and the entire medium was changed every 2 days. For passage, organoids were removed from Matrigel™ and mechanically dissociated into single-crypt domains, and then transferred to fresh Matrigel™. Passage was performed every 1–2 weeks with a 1:4 split ratio. An example of a time-course evolution of jejunum organoids is shown in *Picture 3*.

- **Human cell lines**

Caco-2 cells (human colon adenocarcinoma cells) (passages 30-50) and T84 cells (human colon carcinoma cells) (passages 20-40) were obtained from the ECACC (*ECACC 09042001* and *ECACC 88021101*). They were cultured in Dulbecco´s Modified Eagle Medium (DMEN) or DMEN-F12, (25 mM glucose) supplemented with heat-inactivated fetal bovine serum (10 % v/v), 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 2.5 µg/ml amphotericin and 2mM L-glutamine, in a humidified 5 % CO_2 atmosphere at 37 °C.

1.2. EXPERIMENTAL ANIMALS AND IBD MODEL

- **Rats**

Female Wistar rats (180–220 g) obtained from the Laboratory Animal Service of the University of Granada (Granada, Spain) were used, housed in makrolon cages (3–4 rats per cage) and provided with free access to tap water and chow diet (*Panlab A04, Panlab, Barcelona, Spain*). Rats were maintained in animal quarters at a constant temperature (22–25 °C) and a 12 h light-dark cycle. These studies were approved by the Ethical Committee of the University of Granada and carried out in accordance with the Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes of the European Union (86/609/EEC).

- **Trinitrobenzene sulphonic acid (TNBS) model of rat colitis**

Colitis was induced according to the method described by Morris et al. [542] with minor modifications. Briefly, rats were fasted overnight and anesthetized with isoflurane. Under these conditions, animals were given 10 mg TNBS dissolved in 0.25 ml of 50 % (v/v) ethanol by means of a teflon cannula inserted 8 cm into

the anus. Rats from the non-colitic (control) group received 0.25 ml of PBS. The mechanism of TNBS-induced colitis seems to involve reaction of the hapten with host tissue proteins, generating a variety of new antigens *in situ* as well as the production of free radicals. Rats were kept in a head-down position for an additional 30 s until they were recovered from anesthesia, and returned to their cage. Occurrence of diarrhoea (perianal fur soiling) was recorded daily.

As expected, colonic instillation of TNBS/ethanol triggered an intense inflammatory response that was restricted to the large bowel. This model of colitis has an acute stage lasting 2-3 days characterized by extensive necrosis and ulceration of the distal colon [542-544], followed by a prolonged period of progressive recovery during which the extension of the inflammatory lesion is gradually reduced. This period is approximately 2-3 week long when the dose of 10 mg of TNBS rather than the 30 mg of the original description of the model is used [544]. Thus the colonic status 1 week after TNBS administration represents an intermediate phase of healing from mucosal ulceration and inflammation. Preliminary experiments showed that both the inflammatory status and the alterations in transport were higher at 1 week than at later time points (data not shown). Therefore, all subsequent experiments were carried out 1 week postchallenge.

- **Mice**

*C*ftr$^{-/-}$ mice (Cftr^{m1Cam}, FVB/n) (Cystic fibrosis (CF) mice) [29] and FVB/n littermates (control) were bred and accommodated in an environmentally controlled facility at the Animal Experimental Center of the Erasmus Medical Center, Rotterdam, The Netherlands. Mice were genotyped by polymerase chain reaction (PCR), performed on DNA extracted from toe clips. Mice were housed in a light-controlled (lights on 6 am to 6 pm) and temperature-controlled (21 °C) facility and were allowed access to tap water and a semisynthetic chow diet (*SRM-A; Hope Farms BV Woerden, The Netherlands*) from weaning. All experiments were performed on female and male animals of 10–20 week of age. Experimental protocols were approved by the Ethical Committee for Animal Experiments of the Erasmus Medical Center.

1.3. CONTROL AND IBD HUMAN BIOPSIES

Colon biopsies were obtained at the Hannover Medical School from individuals subject to endoscopy. In addition to regular histology biopsies, 2-3 biopsies from each individual were used for

immunohistochemical evaluation and for electrolyte transport studies. Informed consent was obtained from all subjects participating in the study prior to endoscopy.

Standard multiforceps were used (*Multibite™ Multiple sample Single-Use Biopsy Forceps, Boston Scientific*). Two kind of patients participated in this study: control (with a colonoscopy to discard IBD) and IBD (UC and CD) patients. Total of 23 Patients with IBD (UC and CD patients) and 10 healthy controls were included in the study. Median age was 34 years in the IBD group (7 men, 10 women) and 41 years in the healthy group (4 men, 6 women). The controls were age-matched and had no inflammatory condition of the intestine. These samples were indexed into noninflamed, lightly inflamed and moderately/severely inflamed colitis, according with the histology results from the pathology hospital center, and based on published guidelines [545]. Immediately after the endoscopy, up to three samples were transferred in ice-cold Ringer solution [(in mM): NaCl (115), NaHCO3 (25), CaCl2 (1.2), MgCl2 (1.2), K2HPO4 (2.4), KH2PO4 (0.4), glucose (10)], for immediate mounting in Ussing chambers. One biopsy was stored in 4 % (w/v) buffered paraformaldehyde solution for subsequent histological analysis.

2. EXPERIMENTAL TECHNIQUES

All the chemicals were obtained from Sigma Chemical (Madrid, Spain) unless otherwise stated.

2.1. DETERMINATION OF ELECTROLYTIC TRANSPORT IN USSING CHAMBERS

The voltage clamp technique (`voltage clamping´) allows monitoring electrogenic active transport of ions across biological membranes, in this case through the colonic epithelium. The technique was introduced by Hans Ussing and Karl Zerahn in the 50s, originally to study electrogenic Na^+ absorption by the frog skin [546]. For this purpose, a new device called Ussing chamber, was designed. We have used Ussing chambers in this thesis to study the disturbances in ionic transport from colon rats, human intestinal epithelial cells and biopsies from human colonoscopies.

- **USSING CHAMBER: Short explanation**

The Ussing chamber is composed of two symmetric hemichambers that can hold a piece of tissue (or a cell monolayer) in between [547]. The chambers are coupled to a U-shaped tubing system, made of glass

that is filled with the experimental solution. The tubing is heated with fixed temperature (37 °C) and gassed with carbogen (95 % O_2 / 5 % CO_2) to oxygenate the liquid contents and stir the liquid to ensure complete convection. The lumen of the tubes is connected to the chambers. This U-shaped tube secures an equal hydrostatic pressure on both sides of the chamber and thus, avoids damage caused by bending of the tissue. To avoid a mechanical leak between the two halves, the tissue is stretched using needle pins inserted around the open `mouth´ of one chamber with matching pinholes in the other chamber. This technique is used in rat´s samples. For use of very small tissue samples such as colonic biopsies (exposed area= 0.018 cm^2) a small circular disk that holds the tissue is inserted between the two half-cells once everything is prepared. Cells can be studied also using permeable supports Transwells™ (*Costar Corning*). The chamber includes two pairs of platinum electrodes with saline bridge formed by 4 % (w/v) agar in 3 M KCl solution, which constantly determining the difference potential on both sides of the membrane, and a second pair of electrodes capable of transmitting a continuous electrical current sufficient to cancel said potential difference, located at both sides of the tissue; one pair, situated in close proximity to the sample, is used to measure voltage or potential difference. The second pair apply an electrical current in order to modulate the potential difference at will. Both pairs of electrodes are connected to an electronic device, the voltage clamp apparatus. When the experimental voltage is established artificially the tissue is said to be `voltage-clamped´. The electrodes must undergo maintenance every 2-3 weeks. Normally this voltage is fixed to zero (short-circuited), and under these conditions (which include also the absence of chemical, hydrostatic and osmotic gradients) all passive transport across the preparation ceases by definition and only active transport is maintained. The magnitude of the current needed to nullify the tissue voltage, called the short-circuit current (Isc), is equivalent to the overall active electrogenic movement of ions from one side of the chamber to the other [548, 549]. In the intestine, chloride and bicarbonate secretion makes the luminal or apical side negative with regard to the basolateral, or serosal side under open circuit conditions. In the distal colon, where electrogenic Na^+ absorption is also present, it contributes to this negative potential. When the tissue is short-circuited, a positive Isc is applied automatically.

- **Samples preparation**

To carry out the experiments, we had three kinds of samples: Distal colon of control (non colitic) and TNBS rats, human cells (T84 and Caco-2 cells) and human biopsies (from healthy and IBD patients).

  - **Animals:** the rats were sacrificed with an overdose of isoflurane and the large intestine was obtained and gently flushed clean with ice-cold Ringers solution. The serosa and the outer

muscle layers separated by blunt dissection. The colon was cleaned from mesenteric adhesions and fat. Then, using a glass rod inserted into the colonic lumen, serous and the external muscle were dissected by open incision and curettage with wet cotton in Ringer solution. The samples are thus obtained `mucosa/ submucosa preparations´. In another series of experiments `mucosal preparations´ were dissected from mucosa/ submucosa samples, following the technique described by Andrés et al [27]. Colon was extended longitudinally, opened with the mucosa upwards. Staying at all times wet with cold Ringer solution underwent a slight transverse incision. Mucosal layer then was took off very carefully with the help of two curved forceps. These samples constitute the mucosa preparations.

In the colon of colitis rats, it is possible to differentiate three parts: proximal colon, distal colon (adjacent to the necrotic, and 4.5 cm from the anus) and necrotic colon. Only the distal colon was used. In control animals equivalent fragments were used.

- **Cells:** T84 or Caco-2 cells Transwell^{TM} inserts were growth during two weeks and mounted in Ussing chambers (exposed area 1.12 cm^2).

- **Human biopsies:** biopsies from control and IBD individuals were transported to the laboratory in ice-cold Ringer solution and mounted within 30 min. The use of 10 times magnification through a stereomicroscope (*Nikon, Tokyo*) ensured correct mucosa-serosa orientation and appropriate fixation. The selected biopsies were mounted in an Ussing chamber variant version (see below), the Easy Mount Multichannel Ussing Chambers (*Warner Instruments, Izasa S.A. Barcelona, Spain*), with an exposed tissue area of 0.018 cm^2. To finish the assembly, both sides of the tissue were bathed in Ringer solution.

- **Voltage clamp experiments in animals and human cells**

Two different samples were mounted on Ussing chambers, i.e. distal colonic fragments from rats (exposed area: 0.64 cm^2), and insert of T84 or Caco-2 cells, mounted in special Transwells^{TM} Ussing chambers (exposed area: 1.12 cm^2). In both sides of the cell or tissue preparations, the apical side corresponding to the portion of the colonocytes in contact with the intestinal lumen, and the opposite side or basolateral were bathed with the Ringer solution. Under these conditions the preparations were

subjected to monitoring and short-circuited with DVC-1000 voltage clamp devices (*World Precision Instruments, Aston, United Kingdom*). A voltage step of +3 mV was briefly applied every 5 min to calculate tissue conductance (G) by Ohm's Law. The preparations were allowed to equilibrate (20–30 min in rat tissue, 10-15 min in human cells) until stable basal readings of Isc (I_0) and conductance (G_0) could be obtained. After the calibration, the experiments were performed. A concentration response curve to the secretagogue agent carbachol or isobutylmethylxanthine (IBMX) added from a water concentrated solution, was then obtained. The curves were individually fitted with a sigmoidal logistic equation using the Origin 5.0 computer software (*Microcal Software, Northampton, MA*), from which the EC_{50} was calculated.

- **Open circuit in human biopsies with Easy mount Ussing chambers**

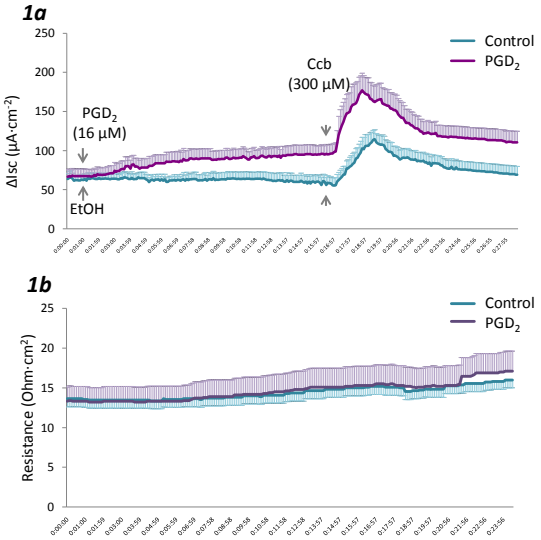


Figure 1a,b. *Tracing of Isc (a) and tissue resistance (b) from control bipsies of patients mounted in Ussing chamber (n=10).*

The human biopsies were mounted in the Easy Mount chambers, which are a two piece assembly using an insert to secure and position tissues. So the biopsy was placed in the chamber by loosening a thumbscrew, sliding the tissue holding insert into the space between the chamber halves, and retightening the thumbscrew. Chambers do not need to be removed from the heater block, nor are the electrodes disturbed, when placing an insert, this is why is called `Easy mount´. Under these conditions the preparations were subjected to monitoring transepithelial potential measurement with EM-CSYS-Ussing chamber devices (*Physiologic Instruments, San Diego, CA*). Current pulses of +1 mV were applied and slope conductance was continuously recorded. Baseline values were recorded after an equilibration period of 15-30 min when stable values were reached. Some of the biopsies did not show these criteria and were excluded from further analysis. Further experiments were performed with different agents. A representative tracing of Isc and tissue resistance from control subjets is shown in *Figure 1*.

Mucosal permeability was examined in some experiments by adding FITC-D 0.1 mg/mL to the apical side of the colonic sheets mounted in Ussing chambers (See below, FITC-D permeability).

A summarize of the reagents, concentrations, side of actuation in the Ussing chamber mountains (apical or basolateral) and action mechanism, is shown in *Table 6*. All regents were obtained from *SIGMA* (*Madrid, Spain*), except L-NIL, from *Cayman chemical,* (*Ann Arbor, MI*), and UFP-512, which was a kind gift of Prof. Gianfranco Baldoni (University of Cagliari, Italy), and we are very grateful.

REAGENT	CONCENTRATION	USSING CHAMBER SIDE	INCUBATION TIME	ACTION MECHANISM
Dd-Ado (2´,5´-Dideoxyadenosine)	200 µM	Apical	15 min	AC inhibitor
Carboxy-PTIO potassium salt	100 µM	Apical/ basolateral	30 min	NO scavenger
L-NIL (L-N6-(1-iminoethyl) lysine)	3 µM	Apical/ basolateral	15 min	iNOS inhibitor
Diethylamine NONOate sodium salt hydrate	100 µM	Apical/ basolateral	30 min	NO donator
Naltrindole hydrochloride	1 µM	Basolateral	30 min	δ-opioid receptor antagonist
UFP-512	1, 10 µM	Apical/ basolateral	15 min	δ-opioid receptor agonist
PGE_2	1 µM	Apical	15 min	$EP_{1\text{-}4}$ receptor agonist
PGI_2 sodium salt	1 µM	Apical	15 min	IP receptor agonist
$PGF_{2\alpha}$ tris salt	1 µM	Apical	15 min	FP receptor agonist
PGD_2	1, 6, 16 µM	Apical	15 min	DP_1 & DP_2 receptors agonist
AH6809	50 µM	Apical	15 min	DP_1, EP_1 & EP_2 receptors antagonist
Ramatronan (BAY-u3405)	0.5 µM	Apical/ basolateral	15 min	DP_2 & TX receptors inhibitor
PTX (Pertussis toxin B oligomer)	200 ng /ml	Apical	20 min	G_i protein inhibitor

Table 6*. Main Ussing chamber reagents, concentration, side of actuation, incubation time and action mechanism.*

All reagents were dissolved in water except naltrindole hydrochloride dissolved in methanol, IBMX and forskolin dissolved in dimethyl sulfoxide (DMSO) and PGs that were dissolved in ethanol. The final concentrations of DMSO and ethanol were at all times lower than 0.1 % without any effect exerted on the preparations.

2.2. FITC-DEXTRAN PERMEABILITY

The Fluorescein isothiocyanate-dextran (FITC-D) (*FD4-16 101287246*) permeability was studied in two kinds of experiments: in distal colon of control rats, and in human cells.

- **Distal colon of rats:**

Paracellular permeability was determined measuring the apical to basolateral flux of FITC-D in mucosa-submucosa preparations from distal colon samples of control and TNBS rats (with or without treatment, (See *Part III.EXPERIMENTAL DESIGNE OF TNBS INDUCED COLITIS IN RAT*) mounted in Ussing chambers. Summarily, after stabilizing the preparation in Ussing chambers (see above), FITC-D 0.1 mg/ml, was added apically to the U-shaped tubing glass at time 0, and after 30 min, 100 µl samples were removed from the basolateral compartment. Fluorescence intensity of each sample was measured (excitation, 490 nm; emission, 520 nm; *FLUOstar-Control, Polarstar OPTIMA, BMG Labtech*). Paracellular flux was calculated as a relative change compared with the control samples.

- **Human cells:**

Paracellular permeability was determined measuring the apical to basolateral flux of FITC-D using a modification of previously described method [550]. Briefly, 1 week post-confluent epithelial monolayer of T84 or Caco-2 cells, on a 1.12 cm^2, 3 µm pore size permeable support (Transwells^{TM}) was washed twice with PBS and maintained at 37 °C. FITC-D, 1 mg/mL, was added apically at time 0, and 50 µL samples were removed from the basolateral compartment at 30 min intervals from 0–420 min, inclusively. Fluorescence intensity of each sample was measured (excitation, 490 nm; emission, 520 nm; *FLUOstar-Control, Polarstar OPTIMA, BMG Labtech*). Paracellular flux was calculated as a relative change compared with the signal of control cells.

2.3. RNA ISOLATION & ANALYSIS OF GENE EXPRESSION BY REVERSE TRANSCRIPTASE-QUANTITATIVE PCR

Different initial protocols were carried out, depending on the nature of the experiment. Thus:

- Rat tissue were harvested, immediately submerged in RNAlater™ and stored over night. After that, tissue was processed for the total RNA isolation.
- Mice and different isolated cells, like colonocytes, crypts and *villi* were processed directly for the RNA isolation.
- Caco-2 monolayers were cultured to confluence as described below and processed directly for the total RNA isolation.
- Mouse small intestinal organoids were cultured for 2-4 weeks, seeded in 48-well plates for the experiments and growth for 2 weeks. When needed, different proinflammatory cytokines (IL-1β, TNFα and IFNγ 1 ng/ml, *eBioscience 14-8012-62, 14-8321-62* and *14-8311* respectively) were added for 24 hours to the culture medium. Then organoids were processed for the total RNA isolation.

In all the cases, the total RNA was obtained by the Trizol method (*Invitrogen, Barcelona, Spain*) and quantity and integrity of RNA were assessed by spectrophotometry (absorbance ratio 260/280 nm) and 1 % (p/v) formaldehyde agarose gel electrophoresis, respectively. Then, 1 μg was retrotranscribed using the iScript cDNA synthesis kit Bio-Rad (*Madrid, Spain*); (In organoids, cDNA was generated from 500 ng). The specific RNA sequences were amplified with *Stratagene MX3005P real time PCR* device using the primers shown in *Table 7a, b, c*.

The PCR program included a pre-incubation step at 95 °C for 10 min to activate the Taq-polymerase prior to 40 cycles of denaturation (95 °C, 30 s), annealing (*x** °C, 1 min) and extension (72 °C, 30 s). The threshold cycle (C_T) of the gene of interest was subtracted from the geometric mean C_T of the housekeeping genes (18s or β-Actin) to yield ΔC_T. The relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method using 18s or β-Actin as a reference genes [551].

*x**: The annealing temperature depends of the primer sequence to amplify. See the table *7a, 7b* and 7*c*.

7a

Homo sapiens gene name	Primer sequence F (5´-3´) *	Primer sequence R (5´-3´) *	Annealing temperature (°C)
Adcy5	TGCTCAGCTTCTACCTGACCTGTT	TGTTCATCTTGGACCGCACGATCT	57
Adcy6	GTGCCCTGTGTTCTTTGT	CTGGATAGTGTGTGCAGATG	56
Adcy9	CCAGCATCGTCAACTTCAGCG	GGTCGTAGCAGTTGAAGTCGC	60
18s	GGACACGGACAGGATTGAC	CGCTCCACCAACTAAGAAC	62

7b

Rattus norvergicus gene name	Primer sequence F (5´-3´) *	Primer sequence R (5´-3´) *	Annealing temperature (ºC)
Adcy5	CATCGATGCCAGGAGTATTG	GGAAACACGACAGGTAGAAG	60
Adcy6	GTTCCAGTCTAAGCAGTTCC	GCTATGTCGGTTACACACTAC	54
Adcy9	GCCAGGAGAAGAAAGAGATG	TCGTACCTGTCGTCTAAGTAT	60
S100a8	GCCCACCCTTATCACCAAC	TGCCCTCAGTTTGTGCAGAA	60
IL-1β	AATGACCTGT TCTTTGAGGCTG	CGAGATGCTGCTGTGAGATTTGAAG	60
CCL2	TCG GCT GGA GAA CTA CAA	CTG AAG TCC TTA GGG TTG ATG	60
MPO	GCCAAACTGAATCGCCAGAAC	ACAGAAGCGTCTCCAGGCATT	60
iNOS	AGCACATTTGGCAATGGAGACTGC	AGCAAAGGCACAGAACTGAGGGTA	57
TNFα	GTC GTA GCA AAC CAC CAA	GCT GAC TTT CTC CTG GTA TG	60
COX-2	TCCAGTATCAGAACCGCATTGCCT	AGCAAGTCCGTGTTCAAGGAGGAT	58
18s	GGACACGGACAGGATTGAC	TCGCTCCACCAACTAAGAAC	60

7c

Mus musculus gene name	Primer sequence F (5´-3´) *	Primer sequence R (5´-3´) *	Annealing temperature (ºC)
Adcy5	GCCAATGCCATAGACTTCA	TCCATCTCCTCCTTCTCTTC	60
Adcy6	GCATTGAGACCTTCCTCATAC	CCGGTTGTCTTTGCTAGAA	60
Adcy9	CTATCGGGATCCATCTGTTTG	CTTGACGGAATTCTCACTCTC	60
Gucy2c	TGTGAACGCGACTTTCATCTAC	GCAGCCCATCTTATGATCTCTTG	60
ATPase α	ACCCAAATGCATCTGAGCCCAAAC	AAGCGTCCTTCAGCTCTTCATCCA	60
ATPase β	ACGAGGCCTACGTGCTAAACATCA	TTGAACCTGCACACCTTCCTCTCT	60
Clca6	AGTCCGTGCAGATGGAGGAAGAAA	TGCTGAAATCCTCCAAGACTGGCT	60
Nhe3	AAGCGCCTGGAGTCCTTTAAGTCA	ATGCTGCTATTCCTCCGCTTCTGT	60
Nkcc1	CTGCTGCAACTGGTATTCT	GTTTGTCAGCTCTGTTGTAATG	60
Cftr	GGAAGATGATGGTGAAGTACAG	AGGCAGAGCTAGTGAAGAA	60
Dra	ACTTTGAAGACAGGTTTGGCGTGG	TATTTGAGGGAGTACACGCTGGCA	60
β-actin	GATGTATGAAGGCTTTGGTC	TGTGCACTTTTATTGGTCTC	60

***Table 7a**. DNA sequence of primer pairs used in PCR assays in human (a), rat (b) and mice (c) tissue.*
**F= forward, R=reverse.*

2.4. MICROARRAY: RNA extraction, microarray hybridization and data analysis

Two mRNA samples were obtained from isolated control and TNBS rat colonocytes (pooled from n=6) as described above. Then, RNA was purified with RNeasy affinity columns (*Qiagen*). Quantity and integrity of RNA were assessed by spectrophotometry and 1 % (w/v) agarose gel electrophoresis, respectively. Sample labeling, hybridization, staining and scanning procedures were carried out using Affymetrix standard protocols (www.affymetrix.com). The microarray analysis was performed by Progenika Biopharma (Bilbao, Spain) on 18 GeneChip® Rat Expression Array 230 2.0 microchips (*Affymetrix®*).

Normalization and statistical analyses were carried out using Affymetrix® GeneChip® Command Console® (*AGCC3.0, Affymetrix®*) and Expression console ™ (*EC 1.1, Affymetrix®*) and the clustering, functional analysis and information about biochemical pathways of the well annotated sequences was achieved using Ingenuity Pathway Analysis (http://www.ingeniuty.com/IPA/), always at $p<0.05$ significance level. Only sequences with annotated gene

identities were considered for analysis. The data were analyzed by analysis of variance followed by Tukey post-hoc tests and Benjamini & Hochberg correction, a procedure envisaged to reduce false positives. Data are expressed as mean±SEM. MIAME recommendations [552] were followed to ensure that all information needed to understand, interpret, reproduce and compare our results was given in detail. Results are expressed as the fold change of the TNBS group versus the control group.

2.5. WESTERN BLOT

The abundance of protein transport related proteins (Na /K ATPase β_1, DRA, Fxyd-4, Claudin-4 and protein $G_{\alpha i}$), PG receptors (EP_2, EP_4, DP_1 and DP_2) and L-PTGD synthase in mucosa-submucosa preparations from intestinal segments, in colonocytes isolated from rats or in Caco-2 cells (confluent 6 well plate monolayer) were measured by Western blot analysis by a standard procedure: tissue or cells samples were homogenized in radioimmunoprecipitation assay (RIPA) buffer (0.1 % w/v SDS, 0.1 % w/v sodium deoxycholate, 1 % v/v Triton X-100 in PBS) with protease inhibitor cocktail 1:100 (v/v). Then homogenates were sonicated and centrifuged at 7000 xg for 5 min at 4 °C. Protein concentration was determined by the bicinchoninic acid assay [553]. Samples were boiled for 5 min in Laemmli buffer, separated by SDS-PAGE and electroblotted to activated PVDF or nitrocellulose membranes (*Millipore, Madrid, Spain*). Then the membranes were probed with the corresponding antibodies. The bands were detected by enhanced chemiluminescence (*PerkinElmer, Waltham, MA*) and quantitated with Image j software. The composition of the Laemmli buffer (5×) was: 312 nM SDS, 50 % (v/v) glycerol, 1 % (v/v) 2-mercaptoethanol, 22.5 mM EDTA trisodium salt, 220 mMTris and traces of bromphenol blue (pH=6.8). Then, samples were transfered to PVDF or nitrocellulose membranes. The antibodies were obtained from: *Santa Cruz* (*Heidelberg, Germany*): NHE3; Na/K ATPase β_1 (N-19); Scl26a3 (DRA) (S-14); FXYD-4 (M-16); PGD2 synthase (T-17) (*Life Technologies S.A. Madrid, Spain*): Claudin-4, *Cell Signaling technology* (*Boston, MA*): G_{α}(i) antibody. *Cayman Chemical* (*Ann Arbor, MI*): EP_2 receptor; EP_4 receptor (C-term); DP_1 receptor; CRTH2/ DP_2 receptor (C-term). As a housekeeping gene, the JLA-20 antibody against actin developed by Lin {Lin, 1981 #854} was obtained from the Development Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biological Sciences at the University of Iowa.

Of note: after the transference, equal loading was checked routinely by reversible Ponceau staining (See Part III of Results).

2.6. HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Biopsies from control and IBD patients were used for histological and immunohistochemical examination. All were fixated in 4 % (w/v) buffered paraformaldehyde over night. They were then dehydrated, embedded in paraffin and cut into 4 μm thick serial sections. For histology, samples were stained with hematoxylin and eosin stain (H&E).

For immunohistochemistry, the sections were deparaffinized in xylene, rehydrated in graded concentrations of ethanol, and the antigen retrival was performed by boiling sections for 20 min in 10 mM trisodium citrate buffer (pH 6.0). Then sections were incubated twice in 3% (v/v) H_2O_2 in methanol for 10 min to block endogenous peroxidase activity. Slides were stained with either polyclonal: anti–DP_1 and anti-CRTH2/DP_2 receptors (1:500 dilution; *Cayman Chemical, Ann Arbor, MI*), and anti-L-PTGDS (1: 50 dilution; *Aviva Systems Biology, BioNova Cientifica, Madrid, Spain*), or monoclonal anti-CD4 (1:100 dilution; *Abcam, Madrid, Spain*) antibodies overnight at 4 °C. After incubation with peroxidase-conjugated secondary antibody (*REAL EnVision Detection System kit, DAKO*), the bound antibody was visualized by avidin-biotin-peroxidase detection using two chromogen kits, diaminobenzidine (DAB) (*REAL EnVision Detection System kit, DAKO*) or aminoethyl carbazole substrate (AEC) (*Invitrogen, Barcelona, Spain*). To finish, slides were counterstained with alum haematoxylin for 2 min, rinsed in water and mounted according to the manufacturer´s instructions of the corresponding chromagen kit.

2.7. SPECIFIC CELL DETERMINATIONS

- **Lactate dehydrogenase (LDH) activity**

LDH is a soluble cytosolic enzyme that is released into the culture medium following loss of membrane integrity resulting from either apoptosis or necrosis. So LDH activity is measured as an indicator of cell membrane integrity and serves as a general means to assess cytotoxicity resulting from chemical compounds. LDH catalyses the reduction of pyruvate by NADH. The rate of decrease in concentration of NADPH, measured photometrically, is proportional to the catalytic concentration of LDH present in the supernatant. For the LDH assay, Caco-2 cells were cultured until confluence and treated with different

proinflammatory cytokines (IL-1β 10 ng/ml, TNFα 10 ng/ml and IFNγ 100 ng/ml) during 24 hours. Untreated cells were used as negative controls and cells treated with triton-X100, 1 % v/v were used as positive control. The supernatants were collected and cleared by centrifugation. LDH was determined by the *LDH-LQ Spinreact kit, BEIS43-1*. All samples were tested in duplicate.

- **Cytokine assay**

For the cytokine secretion assay, Caco-2 cells were cultured until confluence and treated with different proinflammatory cytokines (IL-1β 10 ng/ml, TNFα 10 ng/ml and IFNγ 100 ng/ml) for 24 h. Untreated cells were used as negative controls. Afterwards, the supernatants were collected, cleared by centrifugation at 10000 xg for 5 min, and kept at −80 °C until measurement. IL-8 and MCP-1 were determined by ELISA (*Beckton Dickinson Biosciences, Madrid, Spain*).

- **Adenylate cyclase V gene knockdown**

T84 cells were pretreated with shRNA specific for adenylate cyclase V (AC V) for gene knockdown. All the reagents used in these experiments were obtained from *Santa Cruz Biotechnologies (Heidelberg, Germany*) and the manufacturer's instructions were followed. Briefly, T84 cells were plated on 12 well plates and grown until 50% confluence. T84 medium was supplemented with polybrene^{TM} 5 μg/ml, control and AC V shRNA lentiviral particles, added to the culture medium and incubated overnight. On the third day, fresh medium was substituted for lentiviral particles containing medium and the cells were cultured until confluence for 24 h. Finally, T84 cells were split (1:5), cultured again for 24 h, and selected with a range of 5-10 μg/ml of puromycin dihydrochloride. The knockdown was confirmed by RT-qPCR and western blot, obtaining more than 50 % of downregulation in the mRNA expression and protein levels.

- **cAMP measure by LC–MS/MS analysis**

Caco-2 cells were seeded onto 6-well plates and cultured until confluence. Two kinds of experiments were carried out for the posterior determination of cAMP.

In the first type, the Caco-2 cell monolayers were treated with different proinflammatory cytokines (IL-1β 10 ng/ml, TNFα 10 ng/ml and IFNγ 100 ng/ml) during 24 hours. In the second set of experiments, Caco-2 cells were preincubated with PGD_2 (10 µM) for 5 min, and then treated with IBMX (100 µM) or IBMX (100 µM) combinated with PGD_2 (10 µM) for 10 min. Untreated cells were used in both cases as negative controls.

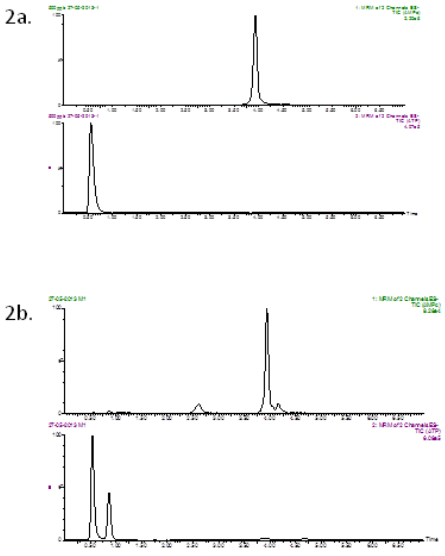


***Figure 2**: chromatogram example of a standard (2a) and sample (2b) for cAMP.*

After both kinds of experiments, the same protocol was carried out: cells were washed with cold PBS, scraped with RIPA buffer with 0.5 mM IBMX and boiled for 5 min at 95 °C. The homogenated were sonicated and centrifuged at 7000 xg for 10 min at 4 °C. Protein concentration was determined in the supernatant by the bicinchoninic acid assay [553]. And the remaining supernatant was stored at -20 °C in injection vials until LC–MS/MS determination. All measurements were completed within 1 week from the time of sample collection.

For the cAMP determination, chromatographic separation was performed using a binary gradient mobile phase consisting of 0.1 % v/v ammoniacal aqueous solution (solvent A) and 0.1 % v/v ammonia in methanol (solvent B). The flow rate was 300 µL/min, the column was maintained at 40 °C and the injection volume was 10 µl. Gradient conditions were: initial mobile phase, 5 % (B), which was linearly increased to 100 % (B) within 4.0 min and held for 3 min to clean the column. Finally, back to 60 % in 0.1 min and kept for 4 min to equilibrate the column. Total run time was 7 min. The mass spectrometer was operated with Electrospray (ESI) in negative ion mode. For increased sensitivity and selectivity, mass spectrometric analyses were performed in SRM (selected reaction monitoring) mode and the MS/MS parameters were optimized individually for each paraben by continuous infusion of 0.5 µg/ml standard solution in the initial mobile phase into the mass spectrometer under combined mode, using $[M–H]^-$ as the precursor ion. Instrument parameters were as follows: capillary voltage, 0.60 kV; source temperature, 150 °C; desolvation temperature, 500 °C; cone gas flow, 150 l/h; desolvation gas flow, 800 l/h; collision gas flow, 0.14 ml/min, and nebuliser gas flow, 7.0 bar. Nitrogen (≥ 99.995%) was used as cone and desolvation gas, and argon

(99.999%) as a collision gas. After selecting the precursor ions, product ions were obtained with a combination of collision energies and cone voltages. The two most sensitive SRM transitions (one used for quantification and the other for confirmation) were selected. For quantification, the most abundant transition was chosen to obtain the maximum sensitivity. Dwell time for each transition was 25 ms, and interscan delay was set at 3 ms. Only one transition was selected for the internal standard because the selected substance is an isotopically labeled compound that is unlikely to be found in environmental samples. Figure 2 shows a chromatogram example of a standard (*2a*) and sample (*2b*) for cAMP. Results are expressed as fmol per microgram of protein.

3. EXPERIMENTAL DESIGNE OF TNBS INDUCED COLITIS IN RATS

3.1. Assessment of colonic inflammation

Female rats (approximately 200 g) were randomly assigned to 4 groups (n= 6), two of which received Ramatroban (10 mg/kg, Sigma, Madrid, Spain) and HQL-79 (1 mg/kg) (*Cayman chemical, Ann Arbor, MI*) orally by means of an esophageal catheter (volume: 2ml). HQL-79 and Ramatroban were dissolved in methylcellulose. A TNBS control group and a saline group were also included for reference. Animals of both groups were given 2 ml of methylcellulose orally. Colonic inflammation was induced in control and treated group as previously described (see above). The administration of ramatroban and HQL-79 was started 2 days before TNBS instillation and continued up to the day before of the sacrifice of the rats, which took place 6 days after the colitis induction. Animal body weight, occurrence of diarrhea (as detected by perianal fur soiling) and total intake for each group were also recorded. Animals were sacrified by overdose of isoflurane and examined for colonic damage. Thus, the large intestine was obtained and gently flushed clean with ice-cold Ringers solution. The colon was cut longitudinally and scored for visible damage on a 0–25 scale according to the following criteria: adhesions (0–3), obstruction (0–2), hyperemia (0–3), fibrosis (rigidity and deformation, 0–5), necrosis (0–5), and other features (proximal dilatation, fragility, scarring (0–4)). The proximal and distal segments of the colon were cut into several fragments. The distal colon fragment adjacent to the necrotic area was mounted in Ussing chambers (see above), to study of permeability with FITC-Dextran and determination of electrolytic transport.

One fragment was kept at −80 °C for myeloperoxidase (MPO) determination and another fragment was processed for the RNA isolation and RT-qPCR determination. Samples were kept at -80 °C for no more than 2

weeks until assay. All biochemical measurements were completed within 2 weeks from the time of sample collection and were performed in duplicate.

Another fragment was processed directly for histological evaluation. Briefly, the samples were fixed in 4 % (w/v) phosphate buffered formaldehyde solution, dehydrated through a graded ethanol series and cast into paraffin wax blocks. Sections (4 μm) were stained with haematoxylin and eosin.

3.2. Myeloperoxidase (MPO) activity

MPO (a marker of leukocyte, mainly neutrophil, infiltration) was measured spectrophotometrically according to the technique described by Krawisz et al. [554]. Samples were suspended in 0.5 % w/v hexadecyltrimethylammonium bromide in Tris (pH=6.0) and minced with scissors for 15 s on an ice-cold plate. The resultant suspension was subsequently diluted to a final 1:20 (w/v) ratio and homogenized for 1 min with an automatic Heidolph homogeniser, sonicated for 10 s and subjected to three freeze-thaw cycles. The homogenates were then centrifuged at 7000 xg (4 °C, 10 min) and the supernatants (100 μl) assayed for MPO activity with 0.0005% H_2O_2 as enzyme substrate and *o*-dianisidine dihydrochloride (533 μmol/l) as chromogen in 3ml 50 mM phosphate buffer (pH=6.0). The enzymatic activity was calculated from the slope of the curve at 460 nm. One unit of MPO activity was defined as that activity degrading 1 μmol H_2O_2/min at 25 °C. Results are expressed as units per milligram of protein.

4. STATISTICAL ANALYSIS

All results are expressed as mean ± standard error of the mean (SEM). Differences among means were tested for statistical significance by either one way analysis of variance and a posteriori least significance test on preselected pairs or nonparametric ANOVA on ranks, depending on the applicable conditions as tested by SigmaStat 3.5 (Jandel Corporation, San Rafael, CA, USA). Differences between pairs of means were analyzed by Student's t-tes7t. Statistical significance was set at $p < 0.05$.

RESULTS

PART I

Before starting to break down the results of this thesis it is important to remark the ion transport situation in IBD: as we have already described in the Introduction, in chronic inflammatory conditions there is inhibition of both ion and water secretion and absorption in the intestine [1, 43, 44, 71, 390]. One of the main goals of this thesis was to study the molecular bases behind these alterations, since these have been poorly described. We had previously demonstrated that cAMP levels in isolated colonocytes are markedly reduced in inflammation [26] and therefore we first aimed to elucidate if there is any relationship between cAMP levels and downregulation of ion transport in IBD.

❖ ***Is the downregulation of ion transport consequence of transportome changes in IBD?***

We used microarray analysis to characterize whether the disturbances in ion transport are due to changes in the expression of transport proteins in enterocytes. We isolated these cells from the colon of rats with colitis induced by the administration of TNBS. The TNBS model of experimental colitis is a widely used model of intestinal inflammation that resembles CD pathology, [555], and it has been used as a primary model for ionic transport disturbances by our group. Actually, the most colitis models, share the same behavior with downregulated basal as well as carbachol evoked secretion [25].

- **TNBS colitis**

Our results indicate that the morphological and biochemical features of TNBS colitis were consistent with previous reports by our group and other authors [391, 542, 543, 556]. Thus, TNBS-treated rats suffered anorexia and loss of body weight associated with extensive mucosal damage, edema, hemorrhage, and early epithelial necrosis. Leukocyte infiltration was prominent, resulting in a significant increase in myeloperoxidase activity. At 5 and 7 days there was major submucosal fibrosis and scarring that resulted in a marked shortening of colonic length.

- **Genomic analysis**

Two mRNA samples obtained from rat colonocytes, namely one control and one TNBS sample (pooled from n=6 each), were compared by microarray analysis. Inflammation changed the expression of 5815 sequences, of which approximately half were upregulated and half downregulated (*Figure 3*).

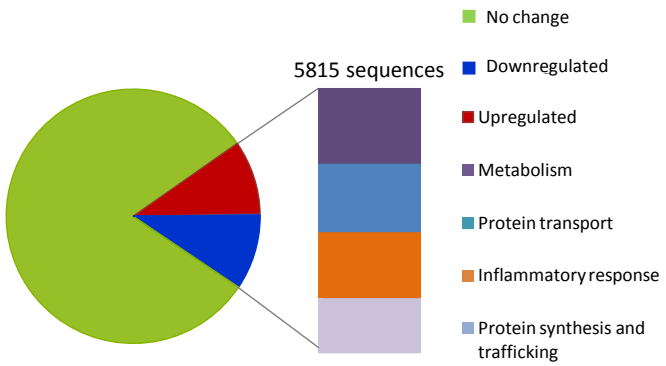


***Figure 3**. Schematic representation of the effect of TNBS on gene expression in rat colonocytes.*

As expected, there was high specific expression of prototypic genes of enterocytes like villin (a major microvillar cytoskeletal actin-binding protein, typically associated with brush border epithelium), the chemokine (C-X-C motif) ligand 1 or Cxcl1 (a chemokine expressed by epithelial cells with neutrophil chemoattractant activity), tight junction proteins (that join together the cytoskeletons of adjacent cells), carbonic anhydrase (CA I and II appear to be good markers for enterocyte differentiation in the colonic mucosa), glucoamylase (a protein present in the brush border region of the enterocyte), lectins, endopeptidase (an integral membrane protein, typically associated with brush border epithelium) or KLF4 (a transcription factor: gut-enriched Krüppel-like factor 4 that plays a role in activating the enterocyte differentiation marker gene intestinal alkaline phosphatase (IAP)) (data not shown)[557].

The colonocyte preparations subject to microarrays were quite pure, although our data indicate the presence of other cells types as indicated by the residual expression of several genes, such as Il1b or Tcrb, reflecting residual contamination with macrophages and T cells. This is considered normal and expected. These genes are upregulated in the TNBS colitis sample, but to a much lower extent than in whole tissue (data not shown), reflecting the enrichment in epithelial cells. Therefore the assessment of nonepithelial specific genes in our analysis must assume a majority, but not exclusively from an epithelial source.

The Ingenuity® platform allowed us the analysis of microarray data and the identification of biological and metabolic pathways affected by inflammation. Our analysis indicated that protein synthesis and trafficking, molecular transport and RNA postranscriptional modification were the main functions affected by inflammation in colonocytes (*Figure 3*).

Our microarray data are in agreement with previous results of our group using full tissue from TNBS colitic rats [557, 558] that showed significant changes directly related to the inflammatory response, metabolism and ion transport. Thus, predictably, the Ingenuity platform found prominent alterations in the inflammatory response. Some of the genes affected are shown in *Table 8* and include genes for cytokines and chemokines like the calgranulin A or S100 calcium binding protein A8 (S100A8), the *monocyte chemotactic protein-1* or Mcp-1(Ccl2), the Growth-regulated protein or Gro (Cxcl2), and inflammatory markers such as lipopolysaccharide binding protein (lbp), nuclear factor of kappa light chain gene (Nfkb1) or toll-like receptor 2 (Tlr2). Metabolism related genes were also highly affected (mostly decreased) by TNBS induced colitis (*Table 8*), including genes participating in glycolysis, like aldolase B (Aldob) and many different subunits of the pyruvate dehydrogenase complex (Dlat or dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex;, Dld or dihydrolipoyl dehydrogenase, Pdha1 or pyruvate dehydrogenase E1 component subunit alpha and Pdhb or pyruvate dehydrogenase E1 component subunit beta). Several enzymes of the Krebs cycle were also changed, e.g., aconitase 2(Aco2),

Gene symbol	C	T vs C	FC T vs C	Change	Gene name
INFLAMMATION					
Pla2g2a	P	P	16.00	I	phospholipase A2 (platelets, synovial fluid)
Il1b	P	P	5.66	I	interleukin 1 beta
S100a8	A	P	3.48	I	S100 calcium binding protein A8, calgranulin A
S100a9	P	P	12.13	I	S100 calcium binding protein A9, calgranulin B
Tgfb1i4	P	P	1.62	I	transforming growth factor, beta 1
Tlr2	A	P	4.29	I	toll-like receptor 2
Xdh	P	P	2.46	I	xanthine dehydrogenase
Ccl2	A	P	2.30	I	MCP-1
Cxcl2	P	P	3.03	I	Gro-d/Cinc-2a/Mip2
Cxcl10	P	P	1.62	I	chemokine (C-X-C motif) ligand 10
Lbp	P	P	2.46	I	lipopolysaccharide binding protein
Nfkb1	P	P	1.62	I	nuclear factor of kappa light chain gene
METABOLISM					
Aldob	P	P	2.30	I	aldolase B
Dlat	P	P	2.00	I	dihydrolipoamide S-acetyltransferase
Dld	P	P	2.30	I	dihydrolipoamide dehydrogenase
Pdha1	P	P	2.00	I	pyruvate dehydrogenase E1 alpha 1
Pdhb	P	P	1.32	I	pyruvate dehydrogenase (lipoamide) beta
Ndufs1	P	P	0.66	D	NADH dehydrogenase (ubiquinone)
Aco2	P	P	1.87	I	aconitase 2, mitochondrial
Sucla2	P	P	1.52	I	succinate-Coenzyme A ligase, beta subunit
Cs	P	P	1.41	I	citrate synthase

***Table 8**. Representative inflammatory & metabolic genes affected by the induction of colitis with TNBS in rat coloncytes. C: control; T: TNBS; vs: versus; FC: fold change.*

succinate-CoA ligase (Sucla2) and citrate synthase (Cs). The respiratory chain gene NADH dehydrogenase (NADH dehydrogenase (ubiquinone) Fe-S protein 1or Ndufs1) and several genes encoding cytochrome isoforms were also downregulated.

Focusing on ionic transport, our data show a major impact of TNBS colitis on a number of genes encoding ion transporters, regulators and related genes. *Table 9a* and *b* shows a summary of these data.

Of note, the main absorptive and secretory transporters, like Nhe3 (Slc9a3) and the ENaC β subunit (Scnn1b) and Cftr (ABCC7) were overexpressed in the enterocytes from colitic rats (*Figure 4, red*), while no changes were observed for Dra (Slc26a3), Nhe2 (Slc9a2) and the ENaC γ subunit (Scnn1g). Therefore, we did not find a correlation

Official symbol	Other aliases	Meaning	Fold
Slco1b2	Oatp-C, Oatp2, Oatp4, Slc21a10, Slc21a6, lst-1	liver-specific organic anion transporter 1	13,93
Slc25a30	Kmcp1	kidney mitochondrial carrier protein 1	11,31
Clca6	Clca4	calcium activated chloride channel	9,19
Slc36a3	Pat3, Tramd2, tramdorin2	proton/amino acid transporter 3	9,19
Slc5a11	Kst1, Rkst1, Sglt6, Smit2	sodium-dependent glucose cotransporter	8,57
Slc12a1	Nkcc2, Bsc1	bumetanide-sensitive cotransporter type 1	8
Slc5a7	Cht1, Hmn7a, hcht	high affinity choline transporter	8
Slco1c1	Bsat1, Oatp14, Slc21a14	organic anion transporter F	8
Slco2a1	Pgt, Matr1, Slc21a2	prostaglandin transporter	7,46
Slco6b1	Gst-1, Tst1	gonad-specific transporter 1	7,46
Slc6a11	Gabt4, Gat3	sodium- and chloride-dependent GABA transporter 3	6,96
Slco3a1	OATP-D, Slc21a11	organic anion-transporting polypeptide D	6,5
Atp1a1	ATPase α1, Nkaa1b	Na+/K+ ATPase 1	6,06
Slc30a1	Znt1	zinc transporter 1	6,06
Slc6a2	Nat1, Net	neurotransmitter transporter	5,66
Slc38a3	Nat1, Sn1, Snat3	N-system amino acid transporter1	4,92
Slc6a3	Dat	dopamine transporter 1	4,92
Slc27a5	Bal, rBal-1	bile acid-CoA ligase	4,59
Slc7a3	Atrc3, Cat3	cationic amino acid transporter 3	4,59
Fxyd5	Kct1	keratinocytes associated transmembrane protein 1	4,29
Slc25a27	Ucp 4	uncoupling protein 4	3,73
Slc7a2	Cat2, Cat2a, Cat2b, Rcat2	cationic amino acid transporter 2	3,73
Slc9a4	Nhe4	Na(+)/H(+) exchanger 4	3,48
Slco1a2	Oatpb1, Oatp2, Slc21a5, Slco1a4	organic anion transporting polypeptide A	3,48
Slc10a1	Ntcp, Ntcp1, Sbact	Na/taurocholate cotransporting polypeptide	3,25
Slc22a3	Oct3	extraneuronal monoamine transporter	3,03
Slc9a3	Nhe3	Na(+)/H(+) exchanger 3	3,03
Scnn1b	ENaC beta	amiloride-sensitive sodium channel subunit beta	2,83
Slc24a2	Nckx2	sodium/potassium/calcium exchanger 2	2,83
Fxyd4	Chif	channel-inducing factor	2,83
Slc28a1	Cnt1	sodium/nucleoside cotransporter1	2,83
Slc6a17	Ntt4	sodium-dependent neurotransmitter transporter NTT4	2,64
Slc7a1	Atrc1, Cat-1	amino acid transporter	2,64
Abcc7	Cftr	cystic fibrosis transmembrane conductance regulator	2,46
Slc30a2	Znt2	zinc transporter 2	2,46
Slc31a1	Ctr1	copper transport 1 homolog	2,46
Slc8a1	Ncx, Ncx1	sodium/calcium exchanger 1	2,46
Slc17a2	Npt3	sodium phosphate transporter 3	2,3
Gnas	G-alpha-8	adenylate cyclase-stimulating G alpha protein	2,14
Slc13a2	Nadc1, mucin	renal sodium/dicarboxylate cotransporter	1,87
Slc27a4	Fatp4	fatty acid transport protein 4	1,87
Slc38a1	Ata1, GlnT, Sat1	amino acid transporter A1	1,87
Slc12a8	Ccc9	cation-chloride cotransporter 9	1,74
Pdzd3	Nhe-Rf4	Na(+)/H(+) exchange regulatory cofactor NHE-RF4	1,74
Atp1b1 /ATPase β	ATPase β	Na (+)-K(+) ATPase subunit beta 1 (non-catalytic)	1,62
Slc35c1	Fuct1	GDP-fucose transporter 1	1,62
Slc31a1	Ctr1, Lrrgt00200	copper transporter 1	1,62
Gnai3	Gip3a, alpha inhibiting 3	guanine nucleotide binding protein	1,52
Slc25a5	Ant2, Atp-adp carrier protein	ATP carrier protein 2	1,52
Slc35a1	Cmp-Sa-Tr	Cmp-sialic acid transporter	1,52
Slc1a1	Eaac1, Eaat3, Reaac1	excitatory amino acid carrier 1	1,52
Slc6a4	5ht transporter	sodium-dependent serotonin transporter	1,52

Table 9a*. Transport genes upregulated by the induction of colitis with TNBS in rat coloncytes.*

between the inhibited ionic transport previously described and the expression of main ion transport genes.

Official symbol	Other aliases	Meaning	Fold
F11r	Jam1	junctional adhesion molecule 1	1,74
Slc11a2	Dmt1, Nramp2	divalent metal transporter 1	1,74
Slc16a6	Mct7B	monocarboxylate transporter 7B	1,74
Slc22a1	Oct1, Orct1, Roct1	organic cation transporter 1	1,87
Slc19a2	Tht1	thiamine transporter 1	1,87
Slco4a1	Oatp-E	organic anion-transporting polypeptide E	1,87
Slc34a2	NaPi-2b	Na(+)/Pi cotransporter 2B	2,46
Slc23a3	Ct1, Octn2, Ust2r	high-affinity carnitine transporter	2,64
Slc22a5	Bmcp1	brain mitochondrial carrier protein 1	2,83
Slc25a14	Lat3	large neutral amino acids transporter subunit 3	3,03
Slc43a1	Mnat, Vmat2	monoamine transporter	3,23
Slc18a2	Ata3	Na(+)-coupled neutral amino acid transporter 4	3,23
Slc38a4	Lyaat1	lysosomal amino acid transporter 1	3,45
Slc36a1	Bsc2, Nkcc1		4,35
Slc12a2	Eaat1, GluT-1	excitatory amino acid transporter 1; glial glutamate transporter	4,55
Slc1a3	claudin-4		4,59
Cldn4	Cnt 2	Na(+)/nucleoside cotransporter 2	4,55
Slc28a2	Pept-2	kidney H(+)/peptide cotransporter	5,00
Slc15a2	Oatp1, Slc21a1, Slc21a3	organic anion-transporting polypeptide	5,00
Slc21a1	Sglt1	Na(+)/glucose cotransporter 1	5,00

Official symbol	Other aliases	Meaning	Fold
Slc5a1	Nckx1	Na(+)/K(+)/Ca(2+)-exchange protein 1	5,26
Slc24a1	Nhe5	Na(+)/H(+) exchanger 5	5,26
Slc9a5	Oat1, Orctl1	organic anion transporter 1	6,25
Slc22a6	Sglt2	Na(+)/glucose cotransporter 2	6,25
Slc5a2	Oatp5a1		6,25
Slco5a1	Oat5, Slc22a19	organic anion transporter 5	6,67
Slc22a19	Nckx4	sodium/potassium/calcium exchanger 4	6,67
Slc24a4	Ncx3	a(+)/Ca(2+)-exchange protein 3	8,33
Slc8a3	VaCht, rVat	vesicular acetylcholine transporter	9,09
Slc18a3	Oatp-d	organic anion-transporting polypep. D	9,09
Slco3a1	Odc1	oxodicarboxylate carrier	11,11
Slc25a21	Clamp, Nherf-3	PDZ domain-containing protein 1	11,31
Pdzk1	Zip2		12,50
Slc39a2	Net	NaCl-dependent norepinephrine transporter	12,50
Slc6a2	Oatp4, Slc21a10	organic anion transporting polypep. 4	12,50
Slco1b2	Octn1	organic cation/carnitine transporter 1	14,29
Slc22a4	Glut1	glucose transporter type 1	20,00
Aqp3		Aquoporin 3	1,52
Aqp8		Aquoporin 8	1,41
Aqp11		Aquoporin 11	1,52

Table 9b. *Transport genes downregulated by the induction of colitis with TNBS in rat coloncytes.*

Nevertheless, the expression of both Na^+/K^+ ATPase subunits α_1 (Atp1a1) and β_1 (Atp1b1) was upregulated, and augmented mRNA levels of Fxyd4 and 5, important regulators of Na^+/K^+ ATPase, were also found (*Figure 4, red*), suggesting a possible regulation of ionic transport at this level i.e. by the regulation of the expression of regulatory proteins. In agreement with this approach, Pdzk1 (Nherf3), the regulatory Pdz-adapter protein of Nhe3, was downregulated (0.09 fold), while Nherf4 (Pdzk3) was upregulated 1.74 fold. Of note, the downregulation of the main Nhe3 regulatory protein agrees with recent results (*Figure 4, blue*) [524, 559, 560].

Finally, several genes involved in epithelial tight junction structure were downregulated, including claudin 4 (Cldn4) and junctional adhesion molecule 1 (F11r) (*Figure 4, blue*). Three aquaporins (Aqp3, 8 and 11) were also repressed in TNBS colitis (0.66, 0.71, 0.66 fold, respectively).

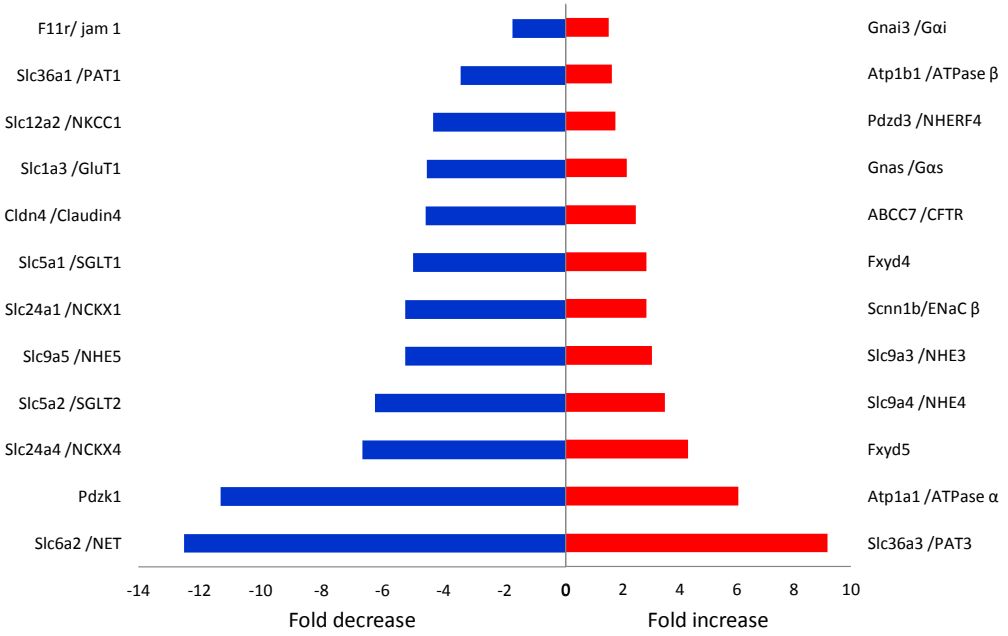


***Figure 4**: Selected transporters, regulators and related genes affected by the induction of colitis with TNBS in rat colonocytes. In red, right side, upregulated transporters and regultors, fold change > 1.5; In blue, left side, downregulated transporters and regulators, fold change <0,6.*

A representative network of PKA signaling pathway is shown in *Figure 5*.

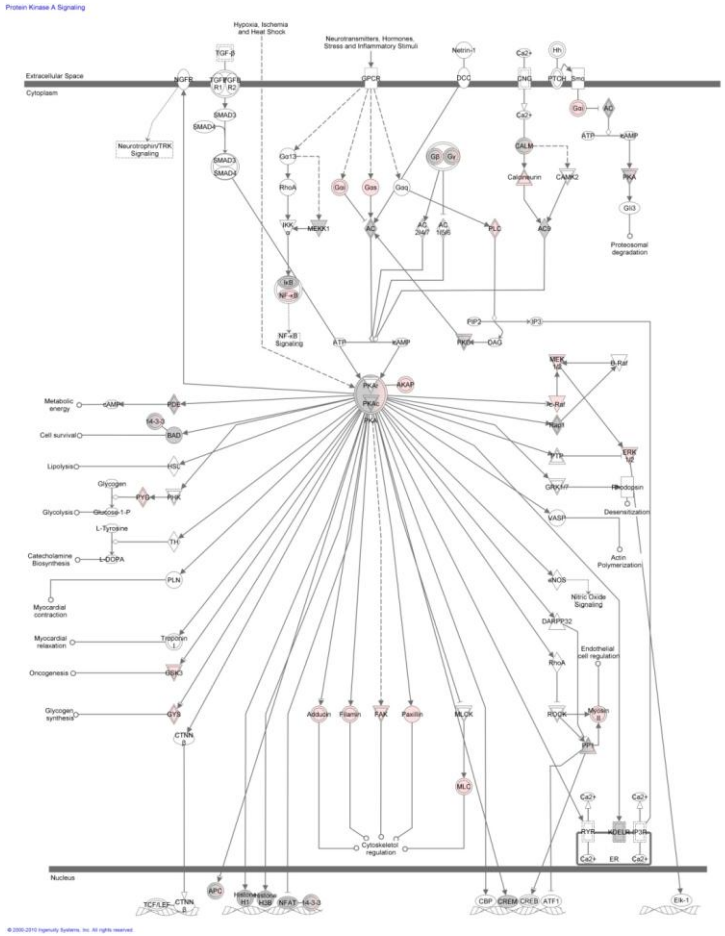


Figure 5. *Genes expression modification related to PKA signaling. Network generated by the informatic Ingenuity® platform.*

Because of the inhibitory state of ion secretion in the inflamed intestine [25], plus the discovery of our group demonstrating isolated colonocytes cAMP levels markedly reduced by inflammation [26], we checked this pathway in the transcriptome analysis, and observed some changes in the cAMP/PKA route. Thus inflammation increased the expression of both G protein subunits $G\alpha_s$ (Gnas) and $G\alpha_i$ (Gnai3) (2.14 and 1.52 fold, respectively) (*Figure 4, red*). In subsequent experiments we tried to study the expression of these two subunits by immunoprecipitation/Western blot, but we had inconclusive results. A summary of these data is shown in the *Illustration 17*.

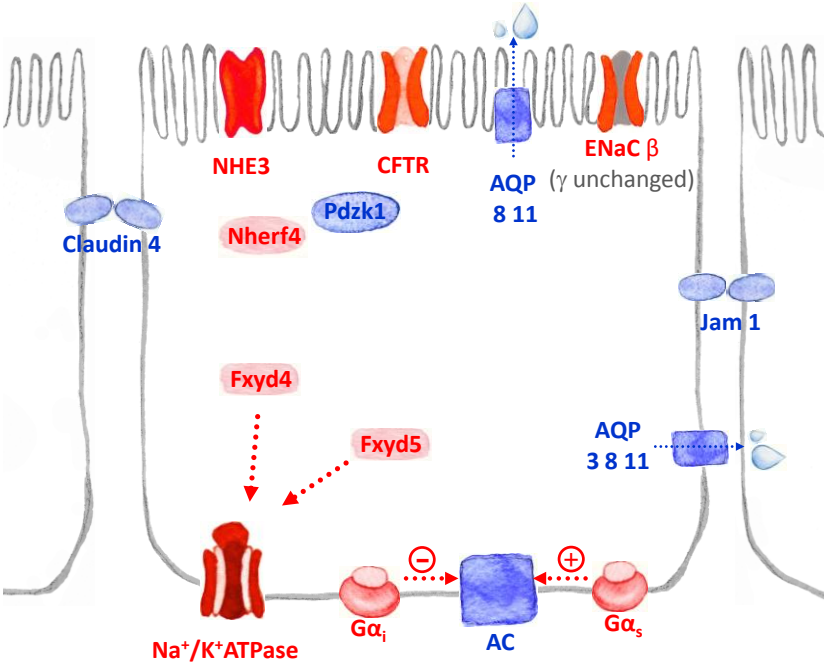


Illustration 17. *Summary of changes in the expression of colonocyte ionic transport related genes induced by the administration of TNBS to rats. Red: upregulated genes in TNBS colitis rats vs control rats. Blue: downregulated genes in TNBS colitic rats vs control rats.*

In addition, we checked the expression of the different isoforms of adenylate cyclase. AC6 and 9 were detected in the microarrays of both control and TNBS colonocytes, while soluble AC (sAC or Adcy10) was detected only in the inflamed cells, suggesting upregulation (*Table 10*).

Of these, only AC9 was found to be present in both samples and significantly downregulated in the microarray (3.4 fold). Because AC5 and AC6 are the two cyclases regulated by Gi protein, we were especially interested in them. AC6 expression was only slightly reduced by inflammation and AC5 was not detected in the microarray. However, it was detected by q-PCR (see below) [413].Phosphodiesterases degrade cAMP, decreasing its levels. Ten phosphodiesterase isoforms were detected in the samples (*Table 11*). Two phosphodiesterase isoforms were found to be upregulated by inflammation (Pde4C and 9A) and three were downregulated (Pde2A, 7A and 6D). It is important to note that Pde4C is the only PDE cAMP-specific, and the major cAMP metabolizing enzyme found in inflammatory and immune cells. Secretion of the gastric hydrochloric acid is assigned to the Pde4 inhibition. Our results indicate an increased

Gene symbol	C	T vs C	FC T vs C	Change*	Gene name
Adcy6	P	P	-0.90	NC	Adenylate cyclase 6
Adcy6	P	M	-1.37	NC	Adenylate cyclase 6
Adcy9	P	P	-3.40	D	Adenylate cyclase 9
Adcy10/ Soluble	A	P	-0.19	NC	Soluble adenylate cyclase

1>*change p-value>0.9955* ***D =Decrease***
0.9955>*change p-value>0.9940* ***MD=Marginally decrease***
0.9940>*change p-value>0.006* ***NC=No change***
0.006>*change p-value>0.0045* ***MI=Marginally increase***
0.0045>*change p-value>0* ***I=Increase***

Table 10*: Adenylate cyclase isoforms detected in microarrays from colonocytes of control and colitic rats. Colitis was induced by the administration of TNBS. C: control; T: TNBS; vs: versus; FC: fold change.*

Gene symbol	C	T vs C	FC T vs C	Change	Gene name
Dpde1	P	P	2.64	I	Phosphodiesterase 4C, cAMP-specific
Pde9a	P	P	4.92	I	phosphodiesterase 9A
Pde2a	P	P	0.76	D	phosphodiesterase 2A, cGMP-stimulated
Pde6d predicted	P	P	0.50	D	phosphodiesterase 6D, cGMP-specific
Pde7a	P	P	0.57	D	phosphodiesterase 7A
Pde3b	P	P	0.44	NC	Phosphodiesterase 3B
Pde8b	P	A	0.11	MD	phosphodiesterase 8B
Pde6h	P	A	0.09	NC	phosphodiesterase 6H, cGMP-specific
Pde4d	A	P	2.00	NC	phosphodiesterase 4D
Pde8a	A	P	1.41	NC	phosphodiesterase 8A

Table 11*: Phosphodiesterase isoforms detected in microarrays from colonocytes of control and colitic rats. Colitis was induced by the administration of TNBS. C: control; T: TNBS; vs: versus; FC: fold change.*

expression of Pde4C in the enterocyte of inflamed rats that could be related to the observed decreased levels of cAMP.

- **Postgenomic validation**

To confirm the changes observed in the expression of AC we carried out a set of RT-qPCR reactions using 3 control and 5 TNBS rat colonocyte samples. AC6 and 9 expression was studied (*Figure 6*). This procedure of independent validation of the results obtained in microarray analysis has become standard in genomic studies. Both isoforms were consistently found to be significantly downregulated (*Figure 6a*). Because AC5, along with AC6, are the two G_i sensitive AC isoforms, as mentioned above, we decided to test also the expression of AC5. Using RT-qPCR, a more sensitive method than microarray, we detected the presence of AC5, albeit at much lower levels than AC9 and 6 (*Figure 6b*). Further, our results demonstrate a downregulation in inflamed colonocytes (*Figure 6a*).

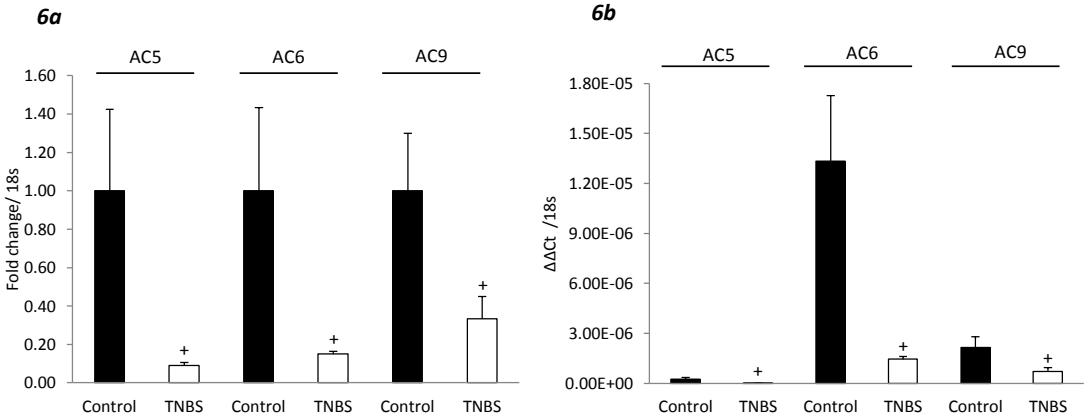


Figure 6a,b. *Absolute (a) and relative (b) expression of adenylate cyclase isoforms 5, 6 and 9 in colonocytes from control and colitic rats. Colitis was induced by the administration of TNBS. Expression was studied by RT-qPCR; Results are expressed as AC fold change related to the expression of the rRNA subunit 18s. C: control. n=3 for C and n=5 for TNBS.*

In an attempt to further describe changes in AC expression by inflammation, we also analyzed the expression of these isoforms in whole colonic tissue (n=6). As in isolated enterocytes, our results show a decreased expression of the three ACs in inflamed tissues (*Figure 7*). However, the expression profile of the three cAMP synthases compared with the epithelial cells is different. Thus, in the whole tissue the profile is AC5>AC9>AC6, while in colonocytes, the ratio is AC6>AC9>AC5.

Additionally, since there is not apparent correlation between the transporter mRNA levels and the actual ion transport situation, we looked at protein expression of the different transporters by Western Blot (*Figure 8*). Although the results failed to show any change, the efficiency of the antibodies was not satisfactory.

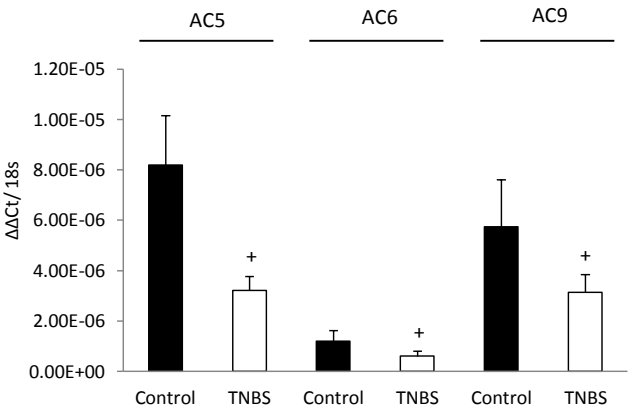


Figure 7*: Expression of adenylate cyclase isoforms 5, 6 and 9 in the colon from control and colitic rats. Colitis was induced by the administration of TNBS. Expression was studied by RT-qPCR. Results are expressed as AC fold change related to the expression of the rRNA subunit 18s. n=6 for control and TNBS.*

In summary, to answer our first question regarding the relation between the downregulation in the ionic transport observed in the intestinal inflammation and the transportome gene expression, we have carried out microarray and postgenomic analysis that show no correlation. Actually, our results suggest that the regulatory proteins may have an important role in these disturbances. Interestingly, previous data from our group showed a decrease in cAMP in the inflamed enterocyte and our results indicate that this could be related to an increased expression of phosphodiesterases and a parallel decrease in specific AC isoforms expression.

PART II

As is already described in Part I of this Results section, we have shown that the downregulation of ion and water transport in the inflamed intestine does not correlate with the disturbances in transportome gene expression (*Illustration 17*). Nevertheless, our microarray data and the postgenomic analysis showed a decreased expression of several ACs (*Figure 6*) that is consistent with the lower levels of cAMP observed in TNBS colitis in rats [26]. To further study the effect of inflammation on cAMP we studied different pathways that modulate cAMP levels in the enterocyte and that could be modified by inflammation. In addition, we tried to elucidate the molecular pathways involved in the observed downregulation of the ACs.

To carry out these experiments, 4 pathways related with cAMP were studied:

A. Proinflammatory cytokines
B. Nitric oxide
C. Opioids
D. Prostaglandins

A. PROINFLAMMATORY CYTOKINES

It is widely known that inflammation is amplified by a broad spectrum of inflammatory mediators, such as IL-1β, TNFα or IFNγ (among others) [39] that are upregulated in IBD [385-387], and these cytokines have been implicated in ion transport alterations in intestinal inflammation [41, 42, 520]. Interestingly, barrier defects may be reproduced in intestinal cell lines with these inflammatory mediators as well [382-384]. Some of these effects may be dependent on changes in the transportome, but regulatory actions are also expected.

The next experiments were designed to study whether there is a link between proinflammatory cytokines, the low expression of AC, and the reduced levels of cAMP in inflammatory conditions.

1. Are cAMP concentration and AC expression regulated by proinflammatory cytokines in an in vitro model?

Because of the difficulties in establishing long-term primary cultures of intestinal epithelium that maintain basic crypt-villus physiology, colon carcinoma cell lines grown as polarized monolayers are frequently used. Thus, the human colon adenocarcinoma cell line Caco-2 was used as a model of intestinal epithelial cells. These cells were incubated 24 hours with three different proinflammatory cytokines: IL-1β, TNFα and IFNγ, to mimic the inflammatory conditions. As *Figure 9a* shows, the addition of these cytokines to Caco-2

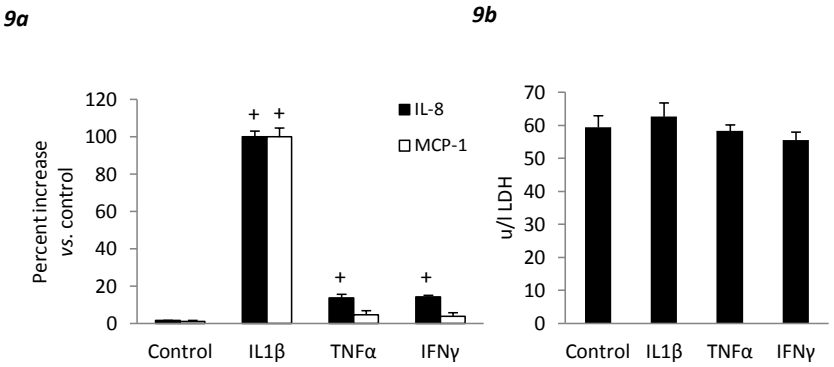


***Figure 9a,b**. Effects of IL-1 β, TNFα and IFNγ on IL-8, MCP-1 (a) and LDH (b)production in Caco-2 cells after 24 h incubation. n=6. + P<0.05 vs. the Control group. Data are expressed as percent increase of the mean ± SEM.*

cells induced the secretion of IL-8 and MCP-1 in the culture media (proinflammatory chemokines that are released by intestinal epithelial cells to recruit inflammatory cells to the mucosa and submucosa during chronic IBD, enhancing intestinal tissue destruction). It should be noted that these three cytokines at the concentration used do not affect the viability of the cells, tested by the measurement of LDH in the supernatant and by the lack of visible pathological changes in the cells (*Figure 9b*).

Consistent with the results obtained with primary enterocytes from TNBS inflamed colon, the stimulation of Caco-2 cells with IL-1β, TNFα and IFNγ resulted in downregulation of AC5, AC6 and AC9 as assessed by RT-qPCR (*Figure 10*). Furthermore, cAMP concentration measured by mass spectrofotometry (*Figure 11*) was significantly reduced after the addition of these cytokines. As expected, the level of ATP, which was measured simultaneously, was unaffected (data not shown). Therefore, our results clearly evidence a direct and acute effect of the different proinflammatory chemokines on the cAMP production mediated by the different isoforms of AC in intestinal epithelial cells.

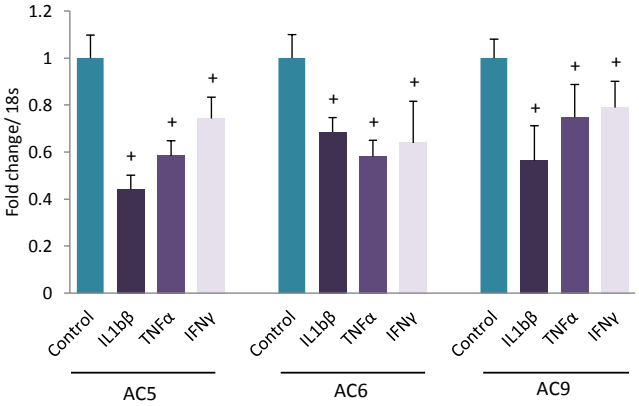


Figure 10. *Effects of IL-1 β, TNFα and IFNγ on the expression of adenylate cyclase (AC) isoforms 5, 6 and 9 in Caco-2 cells after 24h incubation. Expression was studied by RT-qPCR. Results are expressed as AC fold change related to the expression of the rRNA subunit 18s. n=9-10. + P<0.05 vs. The Control group. Data are expressed as mean ± SEM.*

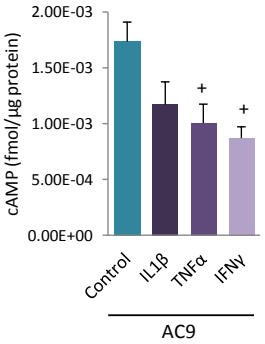


Figure 11. *Effect of IL-1 β, TNFα and IFNγ on the cAMP levels in Caco-2 cells after 24h incubation. cAMP was studied by mass spectrometric analyses. n=5-6. + P<0.05 vs. The Control group. Data are expressed as means ± SEM.*

The next step was to check if the downregulation in the cAMP levels may reproduce the defect in the hydroelectrolytic secretion that is already described in the inflamed intestine. Thus Caco-2 cells were pre-incubated for 24 hours with the proinflammatory cytokines, and mounted in Ussing chambers. After a 15 minute stabilization period, basal Isc and the secretory response evoked by the addition of forsk2olin 10 µM, a direct agonist of AC, was measured. As *Figure 12a* displays, the Isc of the treated cells was decreased compared to that of control cells. Basal secretion (initial Isc) was not significantly affected. Interestingly, at the same time we performed a FITC-D permeability assay in the Ussing chamber (*Figure 12b*). Surprisingly, our results show that permeability was reduced, rather than augmented, by the cytokines (non significant for IL-1β). In contrast, basal conductance is increased in response to IL-1β.

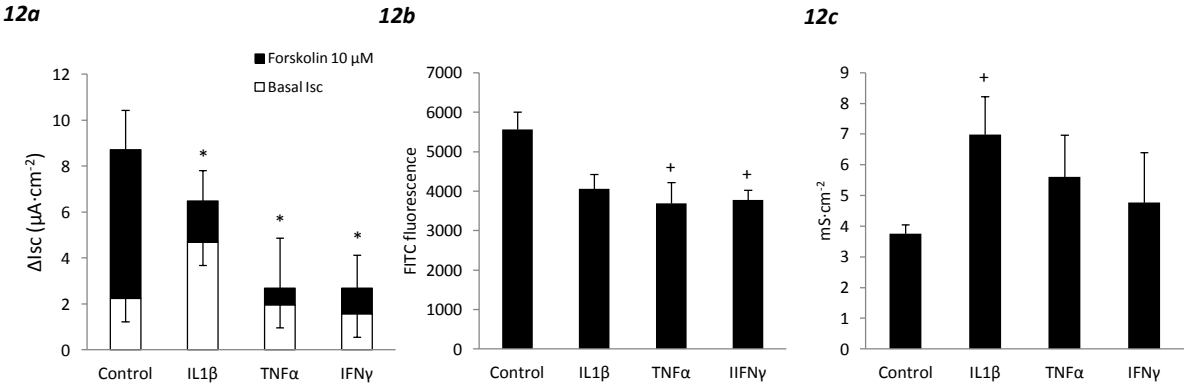


Figure12a-c.*Effects of IL-1β, TNFα and IFNγ on Caco-2 cells incubated for 24 h and mounted in Ussing chambers on: (a) basal and forskolin (10 µM) stimulated secretion; (b) the paracellular permeability to FITC-Dextran; (c) the conductance. n=4-8. * P<0.05 vs. the Isc carbachol response of the Control group. + P<0.05 vs. the control group. Data are expressed as mean ± SEM.*

To verify these last results we performed another permeability experiment *in vitro*, with Caco-2 cells seeded in Transwells™, and the reduction in the permeability of the monolayer was shown again in the treated cells (*Figure 13*).

This set of experiments confirm our results in isolated enterocytes and in tissue and validates the in vitro model used.

A well known established caveat of these experiments is the use of a cancer cell line, since the tumor phenotype differs significantly from that of normal epithelial cells. Thus, their gene expression and polarization pattern may deviate from native enterocytes and colonocytes. Examples are the loss of endogenous NHE3 expression in Caco-2bbe cells, or the complete absence of the CFTR-activating and NHE3-inhibiting enzyme cGMP-dependent protein kinase II (cGKII) in Caco-2 and T84 cells [561]. Therefore we set out to test our hypothesis in a more physiological system.

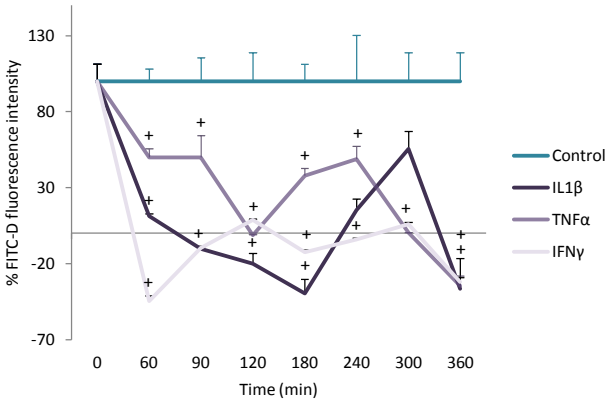


Figure 13. *Effect of IL-1 β, TNFα and IFNγ on the paracellular permeability to FITC-D of Caco-2, measured in Transwells™. n=4-8. + P<0.05 vs. the Control group. Data are expressed as means ± SEM.*

2. Do proinflammatory cytokines have the same effect on intestinal organoids?

Thus far, limited primary cell models are available to study transport disturbances in inflammatory conditions. Recent advances in the identification of Lgr5+ stem cells at the bottom of mouse intestinal crypts allow the growth of crypt-villus structures (´organoids´) in Matrigel™ from isolated crypts or even from a single stem cell in R-Sponding 1-conditioned medium containing Noggin and EGF [540]. This culture technique enables intestinal stem cells to expand into closed organoids containing crypt-like structures and an internal lumen lined by differentiated cells, recapitulating to a large extent the *in vivo* tissue architecture. Thus, to test the effect of inflammation in a third model and specie, closer to the real physiological scenario than Caco-2 cells, we used this novel culture system for mouse small intestine epithelial stem cells [540, 541] (see Material&Metods).

Because our previous results were obtained using the distal colon of rats, or a human adenocarcinoma cell line, and taking into account that the organoids are obtained from mouse jejunum, we first studied and compared the expression profile of AC isoforms in the jejunum and colon of WT mice by RT-qPCR (*Figure 14a*). AC 5, 6 and 9 were expressed in both jejunum and colon, the expression being higher in the latter. We also determined the expression of guanylate cyclase C (GC-G) (*Figure 14*), the main cGMP synthase isoform in mice, since cGMP is a second messenger also involved in transport regulation in the enterocyte with similar effects to cAMP in these cells. Our results indicate that GC-C is also expressed in both sections of the intestine, with higher expression in the colon.

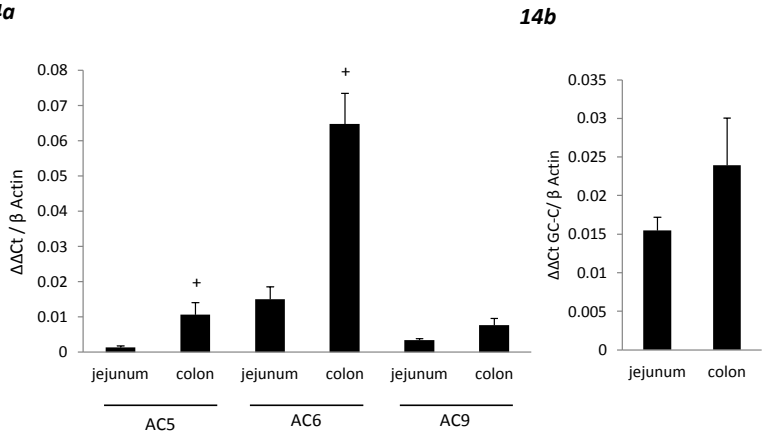


Figure 14a,b. *Expression of adenylate cyclase (AC) isoforms 5, 6 and 9 (a) and GC-C (b) in the jejunum and colon of WT mice assessed by RT-qPCR. Results are expressed as AC fold change related to β Actin expression. n=4-5. + P<0.05 colon vs. jejunum. Data are expressed as mean ± SEM.*

As observed in rats, the main isoform in the mouse intestine is AC6, while the expression of AC5 being very low. To go more into detail, *villi* and crypts from jejunum and crypts from colon mice were isolated, and the distribution of the ACs and GC-C along the crypt-villus axis assessed by RT-qPCR (*Figure 15a-e*). AC5, 6 and GC-C are maximally expressed in the colonic crypts, while AC9 predominates in jejunal crypts. All the AC isoforms and GC-C showed a gradient expression along the villus-crypts, lower in *villi* and higher in crypts.

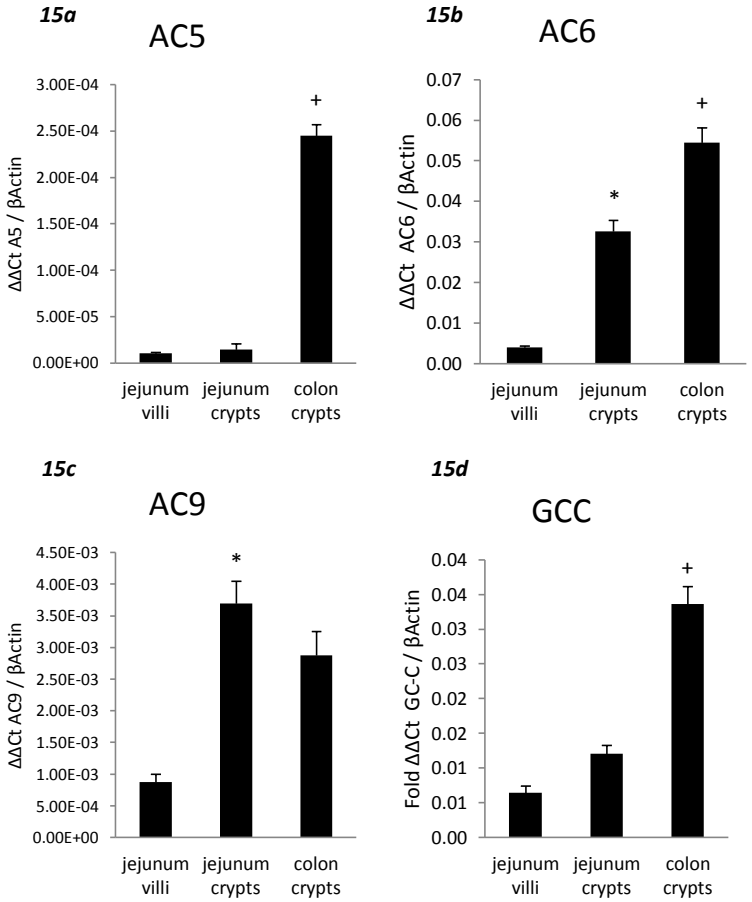


Figure 15a-d. *Expression of adenylate cyclase (AC) isoforms 5, 6 and 9 (a,b,c) and GC-C (d) in jejunum villi, jejunum crypts and colon crypts of WT mice assessed by. Expression was studied by RT-qPCR. Results are expressed as fold change related to β-actin expression. n=4-5. + P<0.05 colon vs. Jejunum. P<0.05 villi vs. Jejunum crypts. Data are expressed as mean ± SEM.*

Next we characterized the expression of ACs and GC-C in the organoids obtained from jejunum crypts of WT mice and cultured in Matrigel™ for one week, and compared it with the expression in the whole jejunum by RT-qPCR. Furthermore, since CF mice were available, we compared the expression of these mRNAs in organoids and whole tissue from these animals with that of WT mice. As shown in *Figure 16a-d*, there was a good correlation between the expression of ACs and GC-C in the organoids and whole jejunum in both WT and CF mice. Our results also indicate that the absence of the Cftr gene in jejunum tissue did not modify the expression of the studied genes.

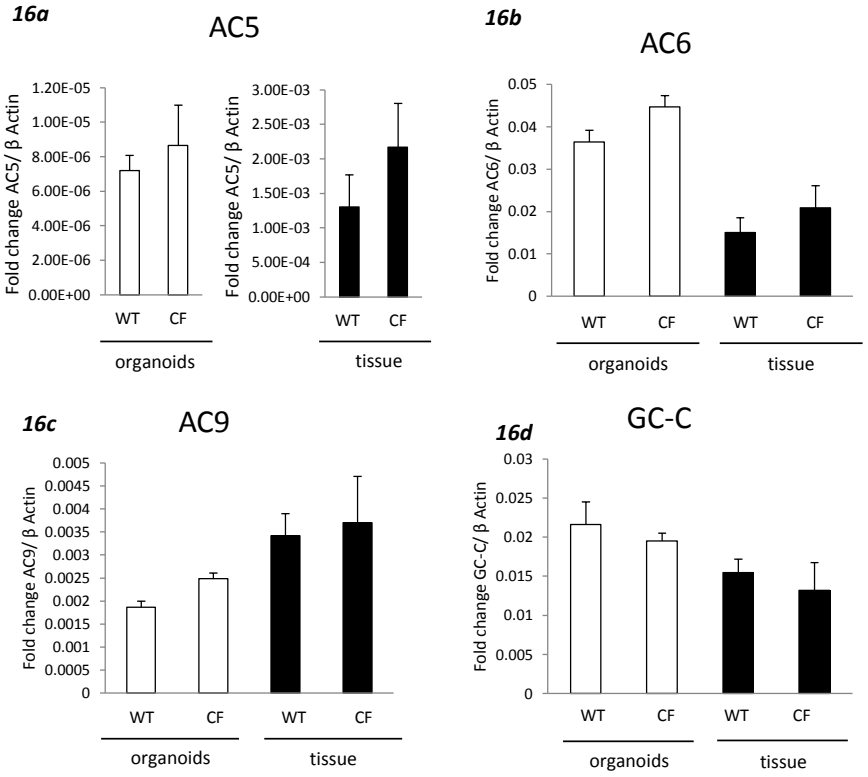


Figure 16a-d. *Expression of adenylate cyclase (AC) isoforms 5 , 6 and 9 (a,b,c) and GC-C (d) in jejunum organoids and jejunum tissue WT and CF mice (Cftr $^{-/-}$). Expression was studied by RT-qPCR. Results are expressed as fold change related to β-actin expression. n=3-5. Data are expressed as mean ± SEM.*

The final aim of our study was to assay the effect of proinflammatory cytokines (IL-1β, TNFα and IFNγ) on the expression of ACs. After incubation for 24 hours of the organoids grown for one week in Matrigel™ with the respective cytokines, a downregulation of the expression CG-C, AC6 and AC9 by TNFα and IFNγ was observed ($p>0.05$ for TNFα and AC5, *Figure 17a and c*). In contrast, the effect of IL-1β was very small or nonsignificant. As we had previously observed AC5 expression was very low, but even so a downregulation by IFNγ could be observed (*Figure 17b)*.

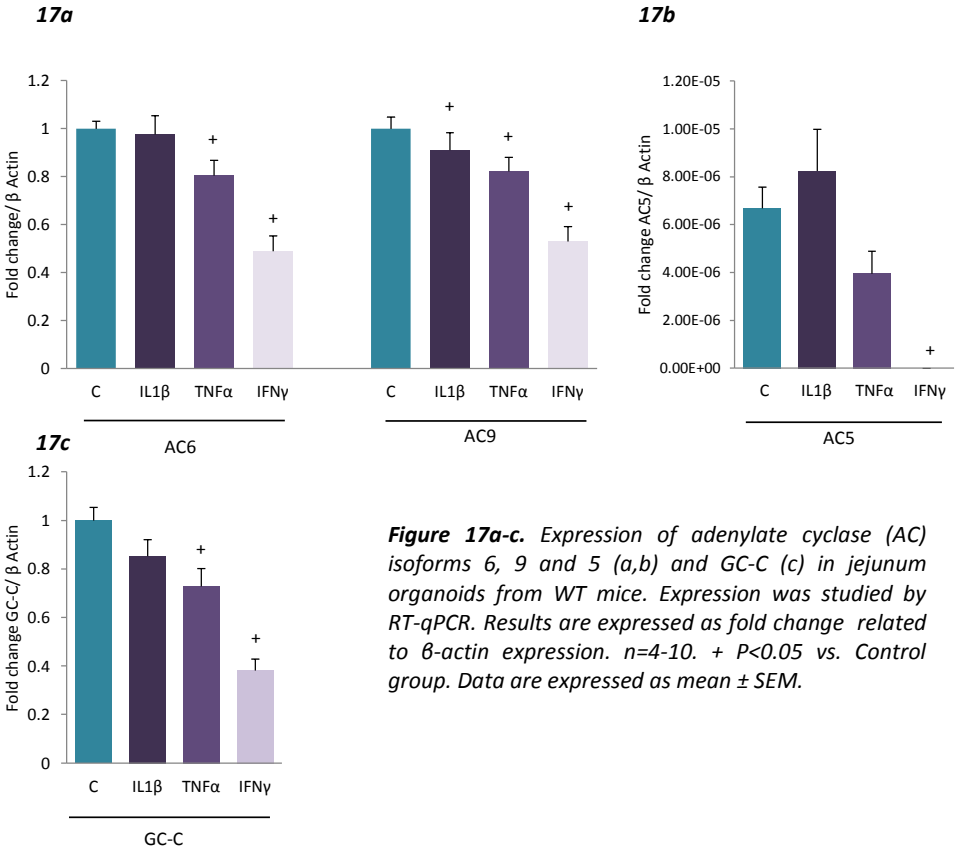


Figure 17a-c. *Expression of adenylate cyclase (AC) isoforms 6, 9 and 5 (a,b) and GC-C (c) in jejunum organoids from WT mice. Expression was studied by RT-qPCR. Results are expressed as fold change related to β-actin expression. n=4-10. + P<0.05 vs. Control group. Data are expressed as mean ± SEM.*

Once we guaranteed that this model may reproduce the alterations we were interested in the expression profile of the different protein transporters was studied. Thus, the expression of the two subunits of Na^+/K^+ ATPase (Atp1a1 and Atp1b1), Cftr (Abcc7), chloride channel calcium activated 6 (Clca6), Nkcc1 (Slc12a2), Nhe3 (Slc9a3) and Dra (Slc26a3) were measured by RT-qPCR (*Figure 18a-g*). In the case of the α1 subunit of Na^+/K^+ ATPase and Dra the three proinflammatory cytokines downregulate their expression, while there were no changes in the expression of the Na^+/K^+ ATPase β subunit. IFNγ reduced the expression of Cftr, Clca6 and Nkcc1, while IL-1β reduced Nhe3. Finally, Clca6 shows significant upregulation with TNFα. These data differ significantly from those obtained with the microarray in colonocytes from TNBS colitic rats (see Discussion).

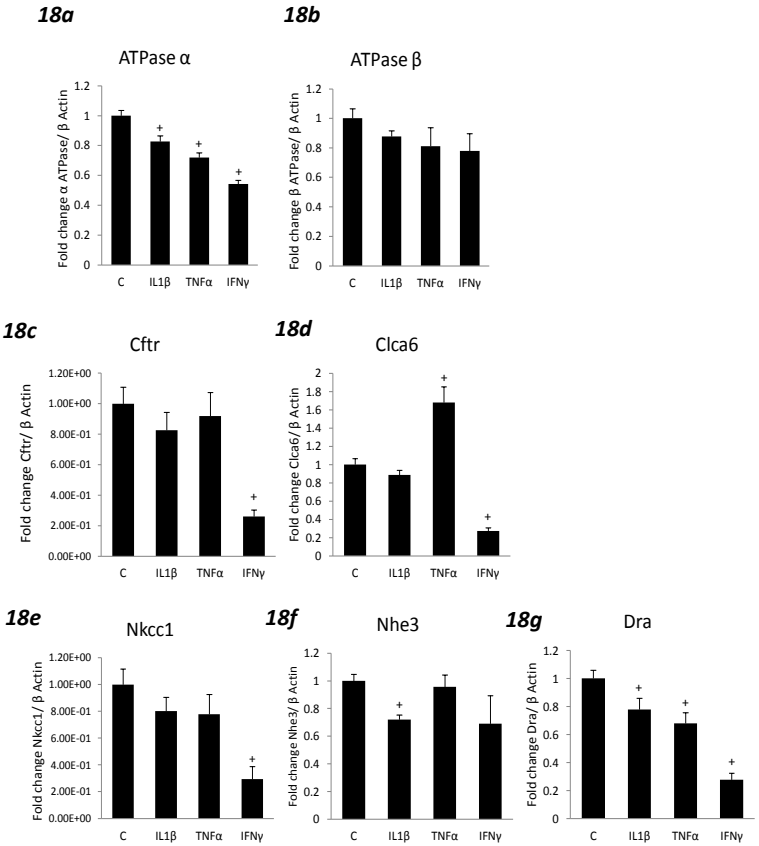


Figure 18a-g. *Expression of ATPase α and ATPase β subunits, Cftr, Clca6, Nkcc1, Nhe3 and Dra in jejunum organoids from WT mice. Expression was studied by RT-qPCR. Results are expressed as fold change related to β-actin expression. n=8-10. + P<0.05 vs. Control group.Data are expressed as mean*

Because CF mice have ample evidence for signs of inflammation in the intestine [562-564], we considered interesting to measure the ACs expression in the colon of WT and CF mice. Indeed, as *figure 19a* and *b* show, we found a tendency towards a decreased the expression of AC5, 6, 9 and GC-C in the colon of CF mice.

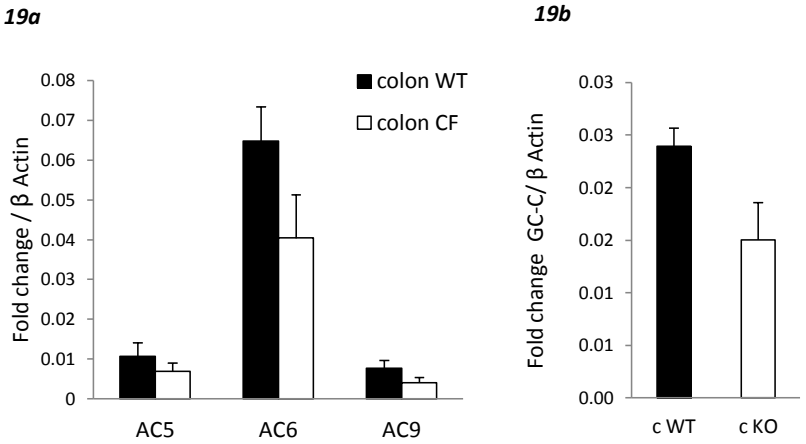


***Figure 19a,b**. Expression of adenylate cyclase (AC) isoforms 5, 6 and 9 (a) and GC-C (b) in colon from WT and CF mice. Expression was studied by RT-qPCR. Results are expressed as fold change related to β-actin expression. n=4. Data are expressed as mean ± SEM.*

We have to point out that we tried to quantify the cAMP levels in organoids, but technical problems prevented a clear and conclusive result. Similarly, we attempted to grow colon derived organoids, but we did not succeed.

To conclude this section, it may be summarized that the disturbances observed in ACs in isolated enterocytes from rat inflamed colonic tissues are reproduced by the addition of proinflammatory cytokines (mainly TNFα and IFNγ) to organoids obtained from mouse jejunum. In addition, the downregulation of AC and GC-C is also observed in the colon of CF mice. However, TNFα and IFNγ exert distinct regulatory effects on transporter expression in this system.

3. Can a defect in AC expression solely reproduce the disturbances of ion secretion in the enterocyte?

To answer this question, and once we knew there were changes in the mRNA levels of the AC, we began checking the activity of the AC, studying the Isc response in the classical cell line utilized in Ussing chamber: T84 human cells derived from colorectal carcinoma. First, we studied the basal and evoked secretion to the secretagogue carbachol in T84 Transwell™ cells pretreated with IFNγ for 24 hours (*Figure 20*). As is already known [565], we observed that IFNγ downregulates the carbachol stimulated secretion in T84. It is important to note that the same result was obtained with the same cytokine in Caco2 cells (see above, *Figure 12a*).

The next step was to study the implication of cAMP synthesis in this downregulation of ion secretion. To this end we knocked down the gene expression of AC5 in T84 cells using shRNA (h) lentiviral particles. A downregulation in stimulated secretion with the secretagogue carbachol was found in these silenced cells (*Figure 21*).

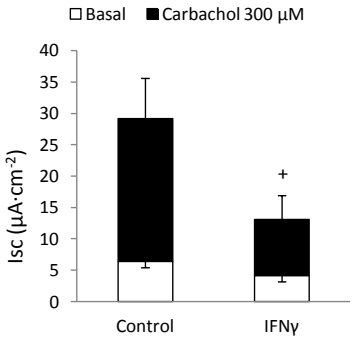


Figure 20. *Effects of IFN-γ (100ng/ml) on basal (white) and carbacol stimulated secretion (black) (Isc) in T84 cells; Isc was meassured in Ussing chambers. n=3-7. + P<0.05 vs. Carbachol response of the control group. Data are expressed as mean ± SEM.*

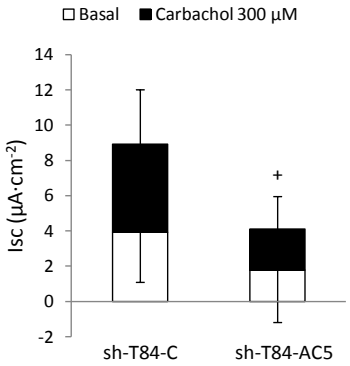


Figure 21. *Basal (white) and carbacol stimulated secretion (black) (Isc) in sh-T84 AC5 cells; Isc was meassured in Ussing chambers. n=5. + P<0.05 vs. Carbachol response of the control group. Data are expressed as mean ± SEM.*

To directly stimulate AC, we carried out the same experiment with the secretagogue forskolin, and a downregulation in basal and evoked secretion was observed (*Figure 22a*). At the same time, FITC permeability experiments were carried out, and we did not found significant changes in permeability in the silenced versus the control cells (*Figure 22b*). No changes in conductance were found either (*Figure 22c*).

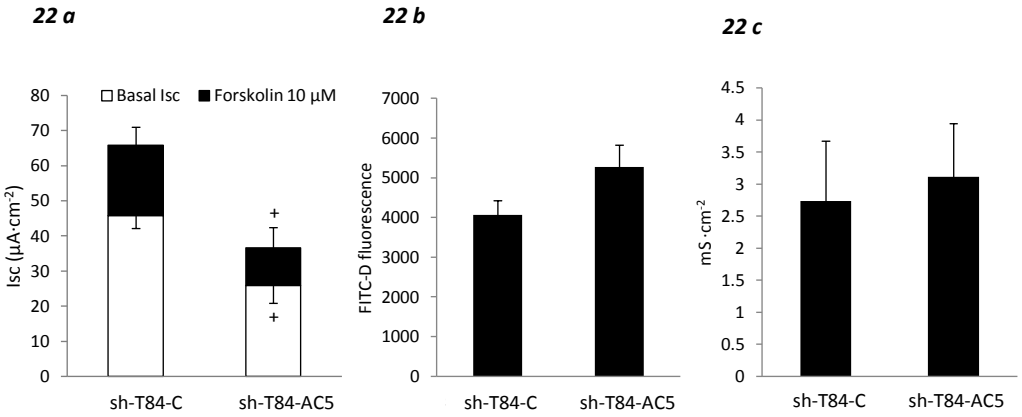


Figure 22 a-c. *Results for T84 cells in wihch de expression of adenylate cyclase 5 (AC5) was silences with shRNA. (a) Basal (whitte) and forskolin (10 uM) stimulated (secretion (black) (Isc); (b) Paracellular FITC-D permeability. (c) conductance. All the parameters were in Ussing chambers. n=8-9. + P<0.05 vs. Control group.Data are expressed as mean ± SEM.*

A second FITC permeability experiment was studied in standard *in vitro* conditions using Transwells™ (*Figure 23*), and again no changes between control and knocked down cells were observed (Data not shown). Preincubation of T84 cells with IL-1β, TNFα or IFNγ for 24 hours showed an increase in the permeability, but no differences were observed between control and AC5 silenced cells (*Figure 23*).

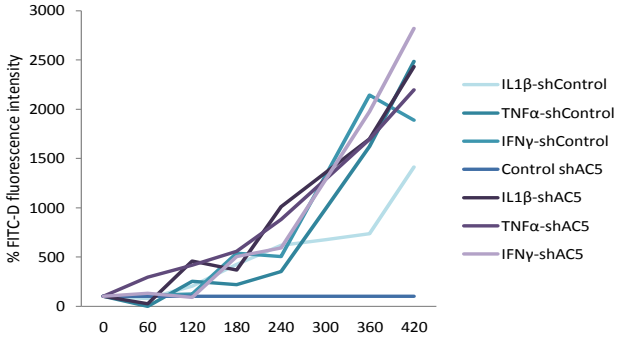


Figure 23. *Effect of IL-1 β, TNFα and IFNγ on paracellular permeability to FITC-D on sh-T84 control and sh-T84 AC5 cells. n=3-4. + P<0.05 vs the control group. Data are expressed as mean ± SEM.*

This set of expreriments indicate that a defective expression in AC5 can by itself reproduce the disturbances of ion secretion observed in the inflamed intestine.

Currently, we are performing new experiments knocking down the other two isoforms (AC6 and AC9), which will give us more information about the role of these two isoforms in the ion secretion of the enterocyte.

4. **Can AC inhibition in the uninflamed rat colon reproduce the disturbances in ion secretion associated with chronic inflammation?**

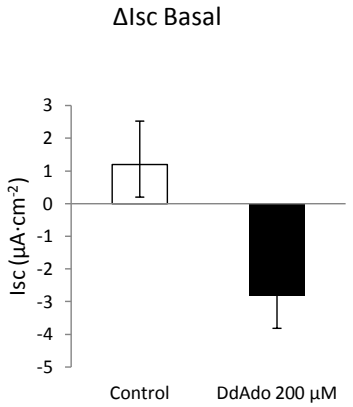


Figure 24. *Effect of DdAdo (200 μM) on basal secretion (Isc). Ddado was added to the apical side of mucosa-submucosa preparations of normal distal colon of rats; Isc was meassured in Ussing chambers. n=8-13. Data are expressed as mean ± SEM.*

Finally, we inhibited AC activity in the distal colon of control (uninflamed) rats to see if this manouver can reproduce the defect in ion secretion observed in the inflamed intestine in Ussing chambers. For that, we used 2',3'-dideoxyadenosine (DdAdo), the most potent (IC_{50}~40 nM) non-protein synthetic inhibitor of AC thus far described. As shown in the *Figure 24*, blockade of AC decreases the basal levels of Isc, which corresponds to a downregulation of ion secretion.

Therefore, AC inhibition results in diminished basal Isc in the uninflamed rat distal colon, comparable to the effect of chronic inflammation.

B. IS NITRIC OXIDE INVOLVED IN THE DOWNREGULATION OF cAMP?

Nitric oxide has been identified as a signaling molecule in the gastrointestinal system [138] and could be involved in the observed downregulation of cAMP, since it has been shown to be a regulator of the basal intestinal water transport. Specifically, there are studies suggest that NO may interfere with cAMP-regulated secretion, including CFTR trafficking, by inhibiting AC in a guanylate cyclase-independent fashion [30].

1. Can a NO scavenger modulate the defective ion secretion in the inflamed intestine?

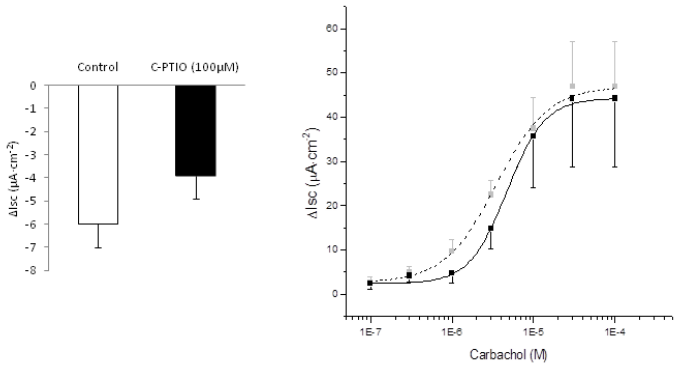


Figure 25. *Effect of carboxy-PTIO (100 µM) on basal secretion (a) and concentration/response curve for carbachol (b) in mucosa-submucosa preparations from inflamed distal colon of rats; Isc was meassured in Ussing chambers; Carboxy-PTIO oxidates NO to yield NO_2 n=6; Data are expressed as mean ± SEM.*

The molecule carboxy-PTIO rapidly oxidates NO to yield NO_2, acting in effect as a NO scavenger. Therefore this molecule has been used to test the importance of NO in various physiological conditions [566]. We used carboxy-PTIO to explore the implication of NO in the downregulation of ionic transport in the inflamed colon of TNBS rats using Ussing chambers. Our results show a non significant trend of the NO scavenger towards the reversion of the reduced basal Isc (*Figure 25a*). No changes were observed in the stimulated Isc secretion curve to carbachol (*Figure 25b*) or in basal conductance (data not shown).

So, removal of NO only shows a trend toward recovery from depressed basal secretion.

2. Is iNOS derived nitric oxide involved in inhibited ion secretion in the inflamed intestine?

L-NIL (L-N6-(1-iminoethyl) lysine) is a potent and selective inhibitor of the inducible NO synthase, iNOS. Our group had already studied in a previous work [26] the effect of L-NIL in the colon of control and TNBS rats. These experiments showed that this inhibitor did not revert the inhibited secretion in the inflamed tissue. However, conflicting results had been reported by other groups [18, 28].Thus we decided to increase the experimental size in order to confirm our results and to boost the statistical power. Our data further confirmed those obtained by our research group and no changes were observed either in basal (*Figure 26a*) or in carbachol evoked secretion (*Figure 26b*).

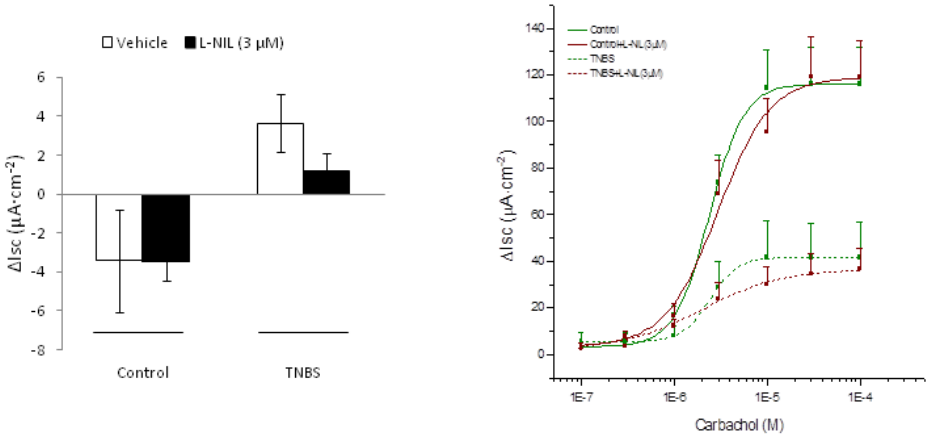


Figure 26. *Effects of L-N6-(1-iminoethyl) lysine (L-NIL) (3 µM), a potent and selective inhibitor of the inducible NO synthase, on basal secretion (a) and concentration/response curve for carbachol (b) in mucosa-submucosa preparations from normal and inflamed distal colon of rats; Isc was meassured in Ussing chambers; n=8-14; Data are expressed as mean ± SEM.*

Thus, iNOS inhibition also fails to revert the downregulation of ion transport in the inflamed colon of rats.

3. Does excess NO reproduce the downregulation of ion secretion characteristic of the inflamed intestine in uninflamed tissue?

Since manouvers leading to a reduction in NO levels in the inflamed rat colon are unable to significantly affect inhibited ion secretion assessed by Isc, we tested the effect of augmenting NO. To this end we used a NO donator, diethylamine NONOate, that in contact with water releases active NO, in the colon of normal rats mounted in Ussing chambers under short-circuit conditions. This resulted in an increase in basal (*Figure 27a*) and carbachol stimulated secretion (*Figure 27b*).

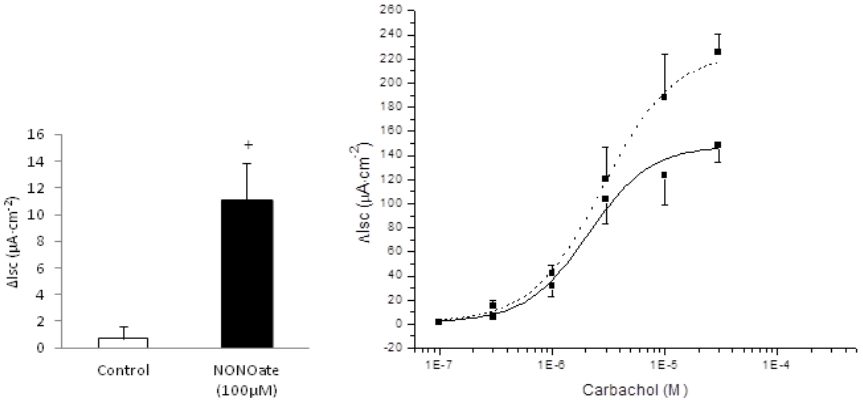


Figure 27. *Effects of NONOate (100 μM), a selective inhibitor of the inducible NO synthase, on basal secretion (a) and concentration/ response curve for carbachol (b) in mucosa-submucosa preparations from normal distal colon of rats; Isc was meassured in Ussing chambers; n=9-12; + P<0.05 vs. Control group; Data are expressed as mean ± SEM.*

Therefore increasing NO in the uninflamed colon enhances rather than inhibits ion secretion.

After this set of experiments in which the involvement of NO has been assesed, we can conclude that NO cannot, at least solely, reproduce the ion transport scenario in the inflamed colon of rats. A minor role is still feasible based on the trend toward Isc recovery after NO scavenging.

C. OPIOIDS

Another pathway that maybe involved in the downregulation of ion secretion through the modulation of the cAMP levels in the enterocyte is the endogenous opioid cascade (Illustration 20). The mechanism of action of opioids include effects on intestinal motility (μ receptors), intestinal secretion (δ receptors), and absorption (μ and δ receptors) [206]. As mentioned in the Introduction, one of the mechanisms of action of the endogenous opioids consists in the reduction of cAMP production by the inhibition of AC though G_i proteins.

1. Are endogenous opioids responsible for the inhibition of ion transport associated with chronic inflammation in the rat colon?

To answer this question naloxone and natrindole were added to inflamed distal colon of TNBS rats mounted in Ussing chambers. Naloxone is a highly selective μ opioid receptor antagonist, but it also inhibits κ and δ receptors. Naltrindole is a highly potent, highly selective δ opioid receptor antagonist [567]. As is shown in Figure 28a and b, naltrindole had any discernible effect on either basal or carbachol evoked secretion. Similar results were observed with naloxone (data not shown).

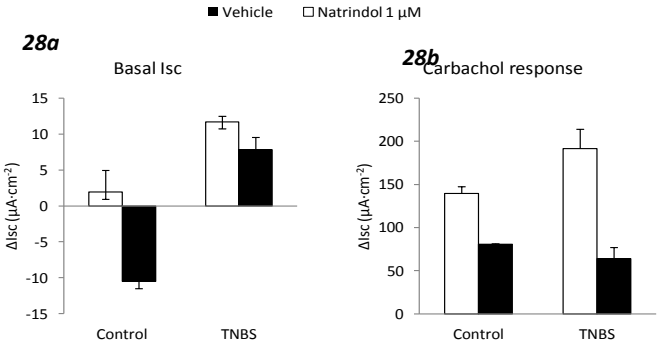


Figure 28a,b. *Effect of natrindole (1 μM), a specific antagonist of δ- opioid receptors, on basal secretion (a) and concentration/response curve for carbachol (b) in mucosa-submucosa preparations from normal and inflamed distal colon of rats; Isc was meassured in Ussing chambers; n=2-7; Data are expressed as mean ± SEM.*

Thus, endogenous opioids are not involved in the disturbances of ion secretion observed in the inflamed intestine.

2. *Can δ-opioid receptors agonists reproduce the transport secretion situation in inflamed colon of rats?*

The next step was to study if the stimulation of the opioid receptors in normal tissue may reproduce the downregulated ion secretion of the inflamed colon. Because the implication of the δ-opioid receptor in the intestinal secretion, we used a novel selective δ-opioid receptor agonist: UFP-512. Thus, we assayed this agonist in the distal colon of control, noncolitic rats in Ussing chambers under short-circuit conditions. As *Figure 29a* shows, the stimulation of the δ-opioid receptor produced a concentration-dependent decrease in basal short-circuit current. In contrast, carbachol evoked secretion was enhanced rather than inhibited in these conditions (*Figure 29b*).

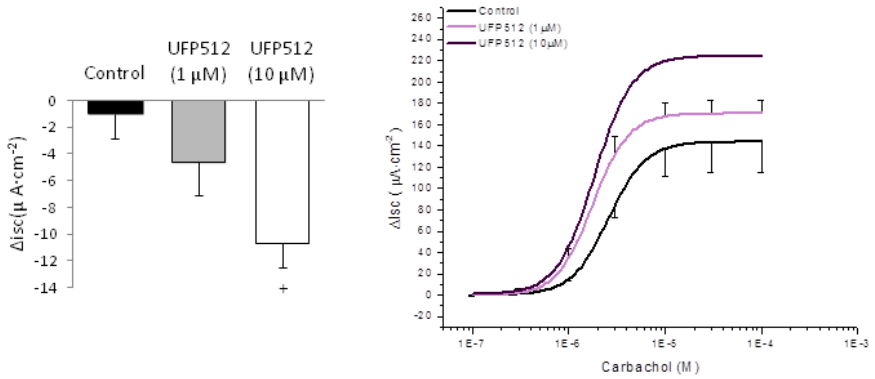


Figure 29. *Effect of UFP-512 (1-10 µM), a specific agonist of δ- opioid receptors, on basal secretion (a) and concentration/response curve for carbachol (b) in mucosa-submucosa preparations from normal distal colon of rats; Isc was meassured in Ussing chambers; n=10; + P<0.05 vs. Control group; Data are expressed as mean ± SEM.*

Thus, activation of δ-opioid receptors can reproduce, at least partially the downregulation of basal secretion that we observe in the inflamed colon. The unexpected and somewhat counterintuitive finding of enhanced carbachol stimulated secretion is however inconsistent with transport in the inflamed intestine. In turn, this finding prompted a explanatory hypothesis that will be presented in the Discussion.

After these opioid results, we can conclude that the endogenous ligands of the opioid receptors do not account for the disturbances in ion transport observed in the inflamed intestine. However, the effect of the δ-opioid agonist on basal ion secretion is consistent in principle with our central hypothesis, i.e. downregulation of epithelial cAMP.

D. PROSTAGLANDINS

As is already described in the Introduction, PGs are regulatory mediators with an important role in inflammation [568]. Inadequate production of proresolving mediators or the inability of these mediators to execute their antiinflammatory effects may exacerbate an inflammatory disorder and could represent an important stage in the progression from acute to chronic inflammation.

PGs exert their actions through a family of GPCRs. All these receptors can modulate AC activity through G_s or G_i proteins, thereby altering intracellular cAMP concentration in the enterocyte (*Illustration 21*). This is why our last block of experiments is focused on the study of these AC modulators, to try to explain the downregulation of the transport situation in IBD.

1. Can PGs be involved in cAMP regulation in the inflamed intestine?

The rat colon has been described to produce both secretory (PGE_2, PGI_2, $PGF_{2\alpha}$) and antisecretory (PGD_2) effects [569-571]. Inasmuch as this role is exerted in the rat colon, a deficient ion secretion may be explained by reduced production or effects of the prosecretory PGs, or by augmented production or effects of the antisecretory PG, i.e. PGD_2.

- **What is the main effect of the different PGs in ion secretion in the distal colon of control rats?**

Before we checked the role of the different PGs in the inflamed intestine, we studied the individual effects of each PG on the normal, uninflamed distal colon of rats in Ussing chambers. As shown in *Figure 30a*, PGE_2, PGI_2 and $PGF_{2\alpha}$ each elicited a significant secretory response in mucosa-submucosa preparations in the absence of inflammation, which was substantially lower in the case of PGE_2 compared with the other two PGs. Conversely, PGD_2 showed a significant antisecretory response. The subsequent response to carbachol was similar in all groups (including vehicle), except PGD_2, which has a paradoxical trend toward an increased maximal response to carbachol, although without reaching the significance threshold (*Figure 30b*). These results indicate that the relative response to this secretagogue is independent of prior PG exposure. Absolute secretion is nevertheless enhanced with the secretory PGs because of the higher baseline.

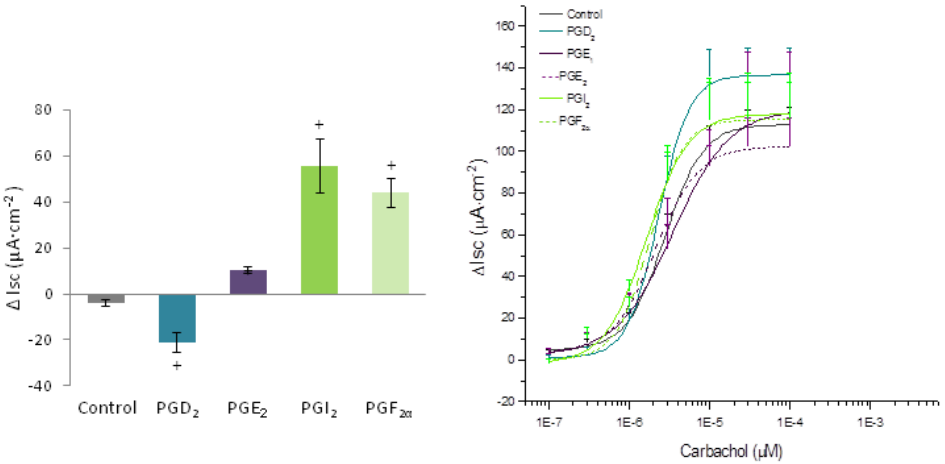


***Figure 30a,b**. Effect of different prostaglandins (PG) on mucosa-submucosa preparations of rat colon. Basal secretion values (a), and concentration/response curve for carbachol (b) are shown. Isc was measured in Ussing chambers. n=5-16. + P<0.05 vs. Control group Data are expressed as mean ± SEM.*

Thus, as expected, PGE_2, PGI_2 and $PGF_{2\alpha}$ have prosecretory effect in basal secretion, and PGD_2 has antisecretory activity.

- **What is the role of the submucosa in the PG effects in rats?**

The submucosa has a pronounced influence on mucosal ion transport, partly because of the continuous production of PGs [572, 573]. Therefore we looked at the actions of the different PGs on the mucosal preparations and compared the results with those obtained with the standard mucosa-submucosa preparations. The PGs with most prominent effects were studied, i.e. PGD_2, PGI_2 or $PGF_{2\alpha}$. As shown in *Figure 31a*, neither PG had a significant effect in basal conditions. Again, PGD_2 showed a tendency toward an increase in carbachol evoked secretion, as occurs in mucosa-submucosa preparations (*Figure 31b*).

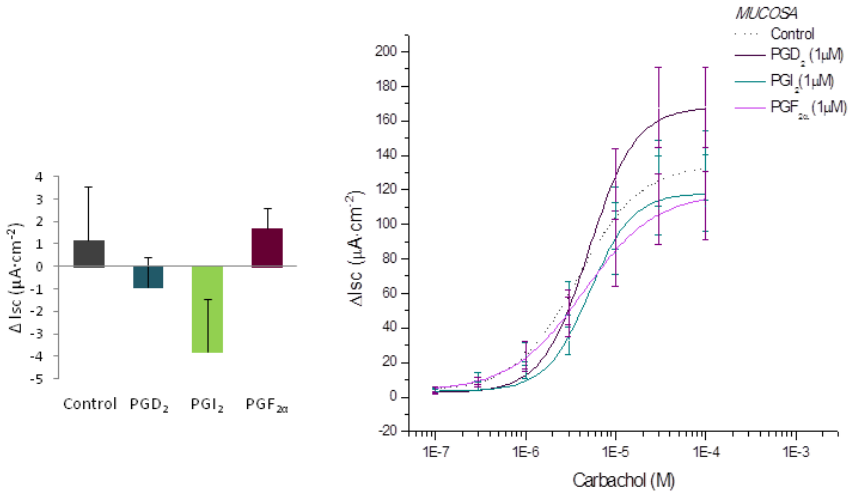


Figure 31a, b. *Effect of different prostaglandins (PG) on mucosa preparations of rat colon. Basal secretion values (a), and concentration/response curve for carbachol (b) are shown. Isc was measured in Ussing chambers. n=5-14. Data are expressed as mean ± SEM.*

Therefore the prosecretory effect of PGs is predominantly indirect, dependent on the presence of the submucosa. Because basal ion secretion of mucosal preparations is very low, it may be very difficult to achieve further significant inhibition, so that the implication of the submucosa in the effect of PGD_2 is uncertain.

- **What is the effect of PGs on ion transport in the inflamed rat colon?**

When the inflamed colon was studied only PGE_2 was able to elicit a response in basal conditions (*Figure 32a*), which was actually comparable to that obtained in normal tissue (see *Figure 30a*). Carbachol evoked secretion was reduced by PGD_2, PGE_2 and PGI_2, while $PGF_{2\alpha}$ did not change the carbachol response (*Figure 32b*).

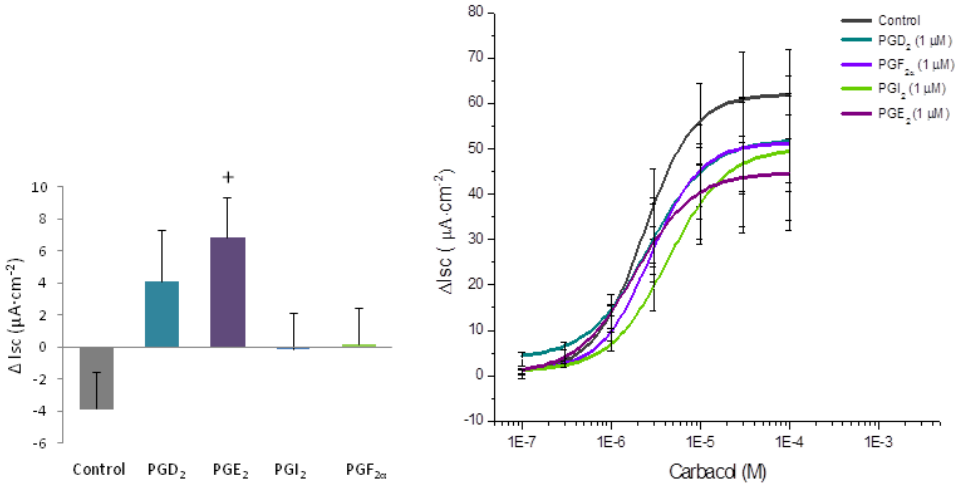


Figure 32a, b. *Effect of different prostaglandins (PG) on colonic mucosa-submucosa preparations from colitic rats. Basal secretion values (a), and concentration/response curve for carbachol (b) are shown. Colitis was induced by the adminstration of TNBS. Isc was measured in Ussing chambers. n=8-17. + P<0.05 vs. Control group. Data are expressed as mean ± SEM.*

Thus the secretory response to PGI_2 and $PGF_{2\alpha}$ was dampened in the inflamed rat colon, while PGE_2 maintained a similar degree of secretion. The antisecretory effect of PGD_2 was similarly lost, indicating that endogenous PGD_2 already produces maximal inhibitory effects or that such inhibition is difficult to appreciate because of reduced spontaneous secretion, as above.

2. Is the attenuation of ion transport in colonic inflammation due to downregulation in the expression of PGE_2 receptors (EP_2 and EP_4)?

PGE_2 has been implicated in the inflammatory process of IBD. Thus, PGE_2 induces the disruption of the intestinal epithelial barrier function through EP_1 and EP_4 receptors [574]. Also, PGE_2 inhibits the migration of colonic lamina propria fibroblasts [575]. As mentioned above, PGE_2 secretory effect appears to be maintained in the rat inflamed colon. Since this PG is increased in intestinal inflammation, including rat TNBS colitis [568, 576], this suggests that lower PGE_2 signaling is unlikely to be involved in depressed secretory responses in the inflamed intestine. To further confirm this point, we measured the levels of the PGE_2 receptors EP_2 and EP_4 by

Name	C	C vs TNBS	Change*	p	Gene name
EP_2	A	P	1.41	NC	Ptger2
EP_4	P	P	0.76	NC	Ptger4

Table12. *Expresion of prostaglandin E_2 receptors $EP_2$and EP_4 in colonocytes isolated from control and colitic rats. Colitis was induced by the adminsitration of TNBS. Enterocytes were isolated, mRNA was extracted and gene expression was studied using microarrays. C: Control group; TNBS: colitic group; A: absent; P: present. NC: no changes.*

Hence downregulation of PGE_2 receptors is not involved in the inhibition of ion secretion associated with chronic inflammation in rat TNBS colitis.

both Microarray and Western blot, comparing control and TNBS colitis derived colonocytes. As *Table 12* shows, EP_2 (Ptger2) is only present in inflamed enterocytes, while EP_4 (Ptger4) is expressed in both, without changes. *Figure 33* displays that EP_2 receptor expression was indeed upregulated in inflamed samples rather than reduced, without changes in EP_4.

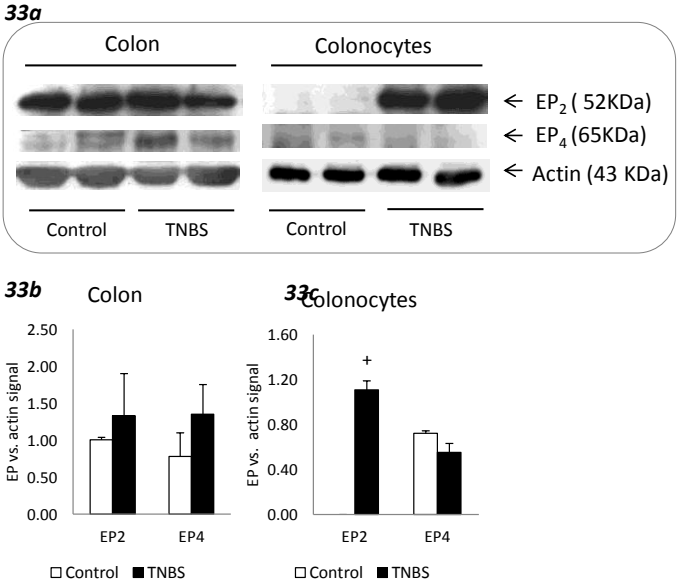


Figure 33a-c. *Representative Western blot for the expression of prostaglandin E_2 receptors EP_2 and EP_4 in colon and isolated colonocytes from control and colitic rats. Colitis was induced by the adminsitration of TNBS. a. Western blot results. b and c. Western blot quantification measured by Image J sofware. Actin was used as a contol housekeeping gene. + P<0.05 vs. Control group.*

3. Is PGD_2 involved in the disturbances in ion transport in the inflamed intestine of rats?

In our previous results, we have demonstrated that PGD_2 has antisecretory effects in basal conditions, but not in carbachol evoked secretion (*Figure 30a*). This is expected to be derived from occupation of DP_2 receptors, which are linked to the activation of G_i proteins, while DP_1 receptors have opposite effects (*Illustration 12*). An enhanced activation of DP_2 receptors at the epithelial level would therefore be consistent with inhibited secretion in the inflamed intestine.

- **Which receptor pathway predominates in the effect of PGD_2 in intestinal epithelial cells?**

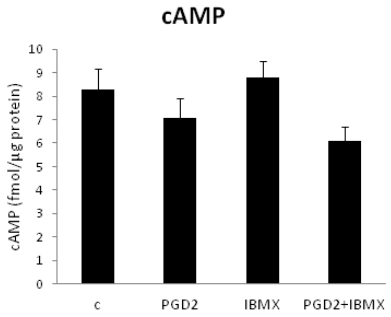


Figure 34. Effect of Prostaglandin D_2 (PGD_2) and the phosphodiesterase inhibitor IBMX on the levels of AMPc in Caco-2 cells. C: control.

While the reduction of basal Isc brought about by PGD_2 in the uninflamed rat colon mounted in Ussing chambers points to a predominant role of the DP_2 receptor at the epithelial level, we aimed to confirm this finding independently. Our first approach was to use immunohistochemistry. However, we could not get rid of high background and therefore decided to discard this option (which was nevertheless useful in human samples, see below). Then we moved to a different system, namely Caco-2 cells, which were exposed to PGD_2 for 10 min, after which cAMP levels were determined. As shown in *Figure 34*, PGD_2 reduced the cAMP concentration inside of the enterocytes, consistent with expression of DP_2 receptors linked to activation of G_i proteins. Furthermore, PGD_2 also prevented the cAMP upshot induced with the phosphodiesterase inhibitor, IBMX.

We can conclude that PGD_2 decreases cAMP in Caco-2 cells, and that intestinal epithelial cells probably express functional DP_2 receptors.

- **Are DP_1/DP_2 receptors expressed in rat colonic tissue? Which is their expression pattern in inflammatory conditions?**

35 a *35 b*

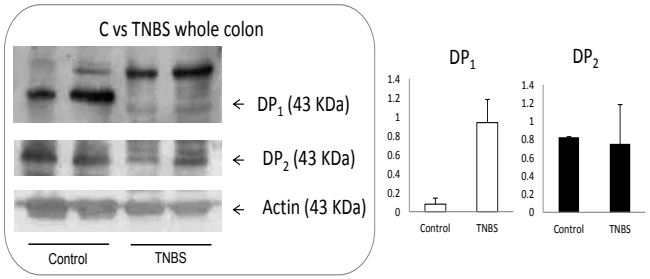


Figure 35 a,b. *Representative Western blot for the expression of prostaglandin D_2 receptors DP_1 and DP_2 in colon from control and colitic rats. Colitis was induced by the adminsitration of TNBS. A. Western blot results. B. Western blot quantification measured by Image J. Actin was used as a contol housekeeping gene*

To study the expression pattern of the two receptors for PGD_2 in rat colon, we assessed the expression of DP_1 and DP_2 in control and TNBS samples by Western blot. We used two types of samples, the whole colon (*Figure 35*) and colon in which the necrotic area was removed (Figure 36). As shown in *Figure 35a and* inflammation decreased the expression of DP_1, while no changes were observed for DP_2 (*Figure 35b*). In the Western blot for DP_1 we observed an increase in the signal of a band with a higher molecular weight in two of the TNBS treated samples. To our knowledge it has not been described in rat that this receptor could dimerize, have such a different glucosilation pattern or an isoform with a higher molecular weight. Therefore we have no explanation for this fact. Nevertheless, it is curious to observe that this effect was also observed when the necrotic area was removed (*Figure 36a, b*). In addition, in these samples there was a decrease in DP_1 and no

36 a ***36 b***

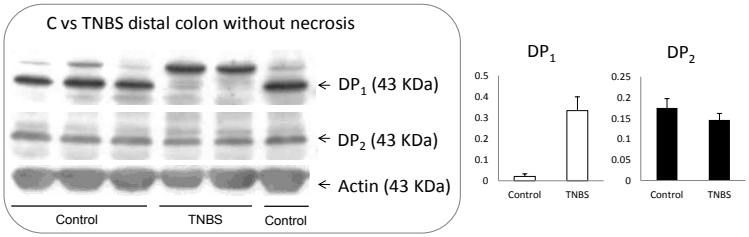


Figure 36 a,b*. Representative Western blot for the expression of prostaglandin D_2 receptors DP_1 and DP_2 in the distal colon in from control and colitic rats. Necrotic areas were removed before tissue homogenization. Colitis was induced by the adminsitration of TNBS. A. Western blot results. B. Western blot quantification by Image J sofware. Actin was used as a contol housekeeping gene.*

changes were shown for DP_2 (*Figure 36a, b*) .

Thus, the inflammation decreases colonic DP_1 protein level in rat, whole no changes are observed in DP_2 . An increase with the inflammation in the expression of higher molecular weight band of unknown indentity in the Western blot for DP1 was observed.

- **Does PGD_2 really enhance carbachol evoked secretion in the rat colon?**

In addition to the inhibition of basal ion transport induced by PGD_2 in the uninflamed rat colon (*Figure 30a*), there was a trend for a higher maximal secretion in the subsequent carbachol concentration response curve (*Figure 30b*). We were intrigued by this, specially in view of the similar effect observed with UFP-512, also an stimulator of G_i proteins (*Figure 29*). Thus, we decided to carry out additional experiments forcing the conditions by increasing PGD_2 concentration from 1 to 6 and then to 16 µM, in order to exclude a possible sensitivity issue, i.e. a false negative result (*Figure 37*). This manouver did not result in a more pronounced decrease of basal Isc (*Figure 37a*), indicating that a maximal inhibition was attained at 1 µM. It also did not reduce carbachol response, but in fact enhanced carbachol induced secretion, as hinted by the original data (*Figure 37b*).

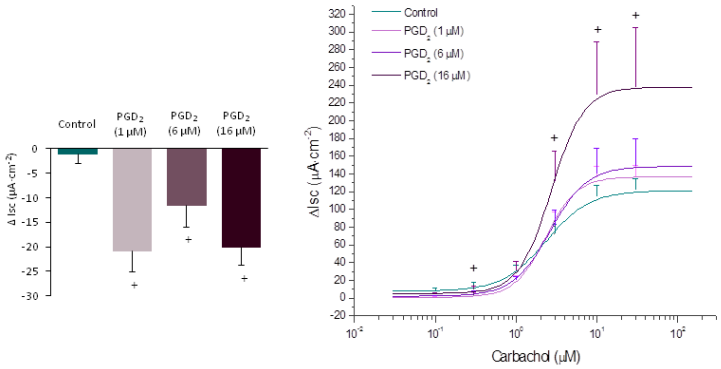


Figure 37,a,b. *Effect of different concentrations of prostaglandin D_2 (PGD_2) on mucosa-submucosa preparations of rat colon. Basal secretion values (a), and concentration/response curve for carbachol (b) are shown. Isc was meassured in Ussing chambers. n=5-16. + P<0.05 vs. Control group. Data are expressed as mean ± SEM.*

Similary, there no differences in the response to IBMX (*Figure 38*).

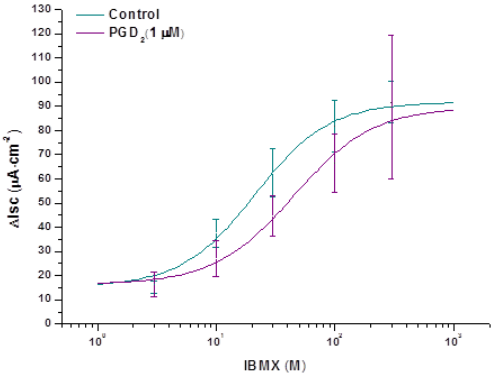


Figure 38. *Effect of prostaglandin D_2 (PGD_2) in the concentration/response curve to IBMX, in mucosa-submucosa preparations from rat colon. Isc was meassured in Ussing chambers. n=7. Data are expressed as mean ± SEM.*

Thus, PGD_2 has a double effect in the uninflamed rat colon, consisting of decreased basal secretion but enhanced carbachol evoked secretory responses, with the latter action being less sensitive than the former.

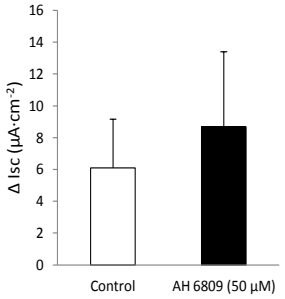


Figure 39. *Effect of* AH6809, an agonist of the prostraglandin D_2 *receptor DP_1 in the basal secretion of the distal colon of colitic rats. Colitis was induced by the administration of TNBS. Isc was measured in Ussing chambers. n= 11. Data are expressed as mean ± SEM.*

- **Is the DP_2 receptor involved in the disturbances in ion transport associated with chronic inflammation in rat TNBS colitis?**

Since PGD_2 can reproduce partly the defect in ion secretion via occupation of DP_2 receptors, our next step was to study its actual involvement in the inflamed intestine. First, as a control to confirm the prevalence of this DP_2 pathway, we tested the effect of AH6809, which is a potent DP_1 receptor antagonist, but also inhibits EP_1 and EP_2 receptors, in the distal colon of TNBS rats mounted in Ussing chambers. As shown in *Figure 39*, AH6809 had no discernible effects, supporting that the inhibitory effect of PGD_2 occurs through DP_2 receptor. Then we used ramatoban, an inhibitor of DP_2 receptors, and also an antagonist of thromboxane receptors. In *Figure 40a* and *b* we can see the effect of ramatroban in the distal colon of TNBS rats, in basal and stimulated secretion with carbachol, respectively. Notably, in the inflamed tissue ramatroban had prosecretory activity in basal conditions, consistent with

a major role of the DP_2 receptor in the inhibited basal Isc in TNBS rat colitis. In addition, there was a tendency toward recovery of inhibition of carbachol induced ion secretion, although without reaching statistical significance.

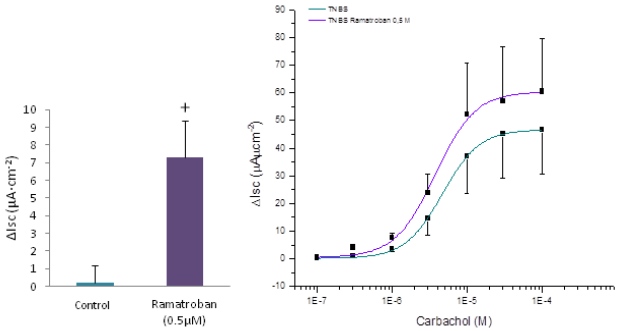


***Figure 40 a, b**. Effect of ramatroban, an antagonist of the prostraglandin D_2 receptor DP_2, in the distal colon rats. Basal secretion values (a), and and concentration/ response curve for carbachol (b) are shown. Colitis was induced by the administration of TNBS. Isc was measured in Ussing chambers. n= 11. + P<0.05 vs. Control group. Data are expressed as mean ± SEM.*

Therefore the DP_2 receptor is involved in the inhibition of ion transport associated with rat TNBS colitis.

- **Is the L-PGD synthase expression modified in inflamed conditions in rat?**

As already described in the Introduction, PGD_2 can be produced by two synthases: L-PGDS and H-PGDS. L-PGDS has been shown to be proinflammatory. Fujitani et al. reported that L-PGDS transgenic mice exhibit strong allergic lung responses and eosinophilia [467] with enhanced allergic airway inflammation. In a model of chronic allergic dermatitis, the blockade of L-PGDS with an inhibitor led to significant attenuation of inflammatory response [442], which was also confirmed in CRTH2 knockout mice. The proinflammatory role of L-PGDS has also been suggested in human UC. Hokari et al. showed that the level of L-PGDS mRNA expression is increased in UC patients in parallel with disease activity [443, 444]. This is why, to study if the effects of PGD_2 are due to some changes in the L-PGD2 synthase, the expression of L-PGD2 in distal colon of control and TNBS rats was assessed by Western blot, and as *Figure 41a* shows, there is an upregulation in the expression of this PGD_2 synthase in the inflamed tissue. This was a significant change (*Figure 41b*).

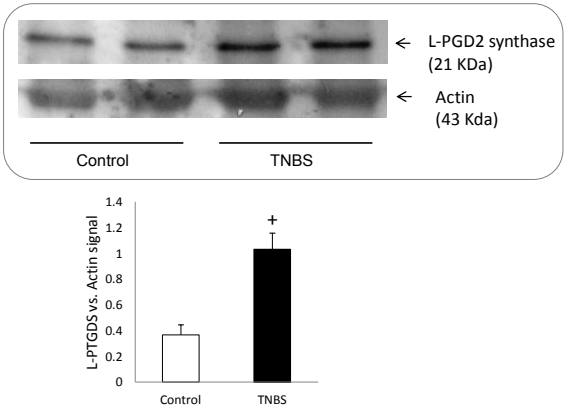


Figure 41. Representative Western blot for the expression of L-prostaglandin D_2 synthase isoform (L-PTGDS) in the distal colon from control and colitic rats. Colitis was induced by the adminsitration of TNBS. a. Western blot results. b. Western blot quantification measured by Image j sofware. Actin was used as a contol housekeeping protein. + P<0.05 vs. Control group.

These results suggest that the inhibitory effect of PGD_2 may be increased in the inflamed intestine due to an overexpression of L-PGD2 synthase.

4. Is PGD_2 involved in the disturbances in ion transport in the inflamed intestine of humans?

- **Which is the effect of PGD_2 in control human biopsies?**

In our previous results, we have demonstrated that PGD_2 has antisecretory effects in basal conditions, and opposite effects in the evoked secretion with carbachol (*Figure 37*). In agreement with these results, the same PGD_2 studies were carried out in human biopsies of distal colon from healthy patients, with a 15 min incubation with PGD_2 (*Figure 42a*), followed by stimulation of ion secretion with carbachol (*Figure 42b*). The information of the number and age of the patients is contained in *Table 13*. An example of H&E stain can be seen in *Picture 2* from the Introduction. Surprisingly, the basal effect of PGD_2 was opposite to that observed in rats. *Thus, PGD_2 has basal prosecretory effects. However, the effect of PGD_2 on carbachol evoked secretion is similar to the effect in rats, with a trend toward an increased carbachol response.*

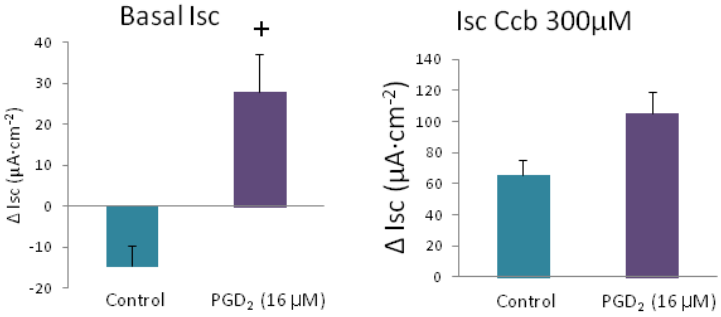


***Figure 42 a, b**. Effect of prostaglandin D_2 (PGD_2) on biopsies from the distal colon of healty humans. To study basal secretion biosies were incubated for 15 min with PGD_2 (a). An additional stimulation of ion secretion with carbachol was performed to obtain stimulated values (b). Isc was meassured in Ussing chambers. n=10.+ P<0.05 vs. Control group Data are expressed as mean ± SEM.*

Groups	Control patients	PGD_2 partients
Nº	10 (6w, 4m)	10 (5w, 5m)
Age	47	36

***Table 13**. Patient's data information: number and age.*

PGD_2 has prosecretory basal effects in the distal colon of human biopsies, opposite to the response in rats, although the impact on carbachol evoked secretion may be different.

- **Could the blockade of DP_2 receptors reverse the inhibition of ion transport in the human inflamed intestine?**

To answer this question we studied the effect of ramatroban in distal colon biopsies of IBD patients. The number and age of the patients is shown in *Table 14.* An example of H&E stain from UC and CD patients can be seen in *Picture 2* from the Introduction. As shown in *Figure 43*, there is a trend to increase the basal secretion in the inflamed biopsies. In order to go into detail, the human biopsies from IBD patients were classified in noninflamed, lightly inflamed and moderately/severely inflamed, according with the histology results from the pathology hospital center.

Groups	Non inflamed	Lightly inflamed	Moderately/ severe inflamed
Nº control patients	10 (5W, 5M)	2 (1W, 1M)	7 (2W, 5M)
Age control patients	35	33	31
Nº ramatroban patients	9 (3W, 6M)	6 (5W,1M)	4 (3W, 1M)
Age ramatroban patients	33.5	37.8	24.3

Table 14. Patient's data classified according to the status of the colon as observed by the person obtainnig the

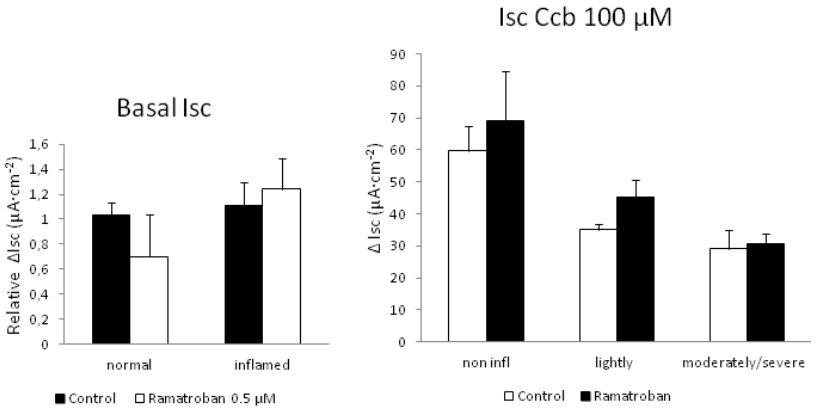


Figure 43 a, b. *Effect of ramatroban, an antagonist of the prostraglandin D_2 receptor DP_2 , on biopsies from the distal colon of IBD patients. Secretion biopsies were incubated for 15 min with ramatroban (a). Patients were divided according to the status of the colon, as observed by the person obtaining the biopsia into non inflamed, lightly inflamed and moderately to severe inflammation?(b). Isc was meassured in Ussing chambers. N=4-10. Data are expressed as mean ± SEM.*

In all the groups there was a tendency toward increase of carbachol stimulated secretion. There were no significant differences however, due to the small number of patients.

- **How does DP_1/DP_2 receptor expression in human colon compare with rat tissue?**

To study the expression pattern of the two receptors of PGD_2 in human colon, we performed an IHC experiment of DP_1 and DP_2, in control and IBD biopsies. *Figure 44a* shows the expression of both receptors in a control tissue, with a submucosal expression of DP_1, and undetectable DP_2 signal. Conversely, in IBD samples (*Figure 44b)* there is a downregulation of DP_1 and marked induction of DP_2 at the epithelial level.

Hence, contrary to the scenario in rats, where DP_2 is expressed in both normal and inflammatory conditions, there is a clear predominance of the DP_1 receptor in the uninflamed human colon, with corresponding prosecretory responses to PGD_2. Under inflammatory conditions however DP_2 is induced and this seems to be consistent with antisecretory modulation of secretion, although more data are necessary in this regard.

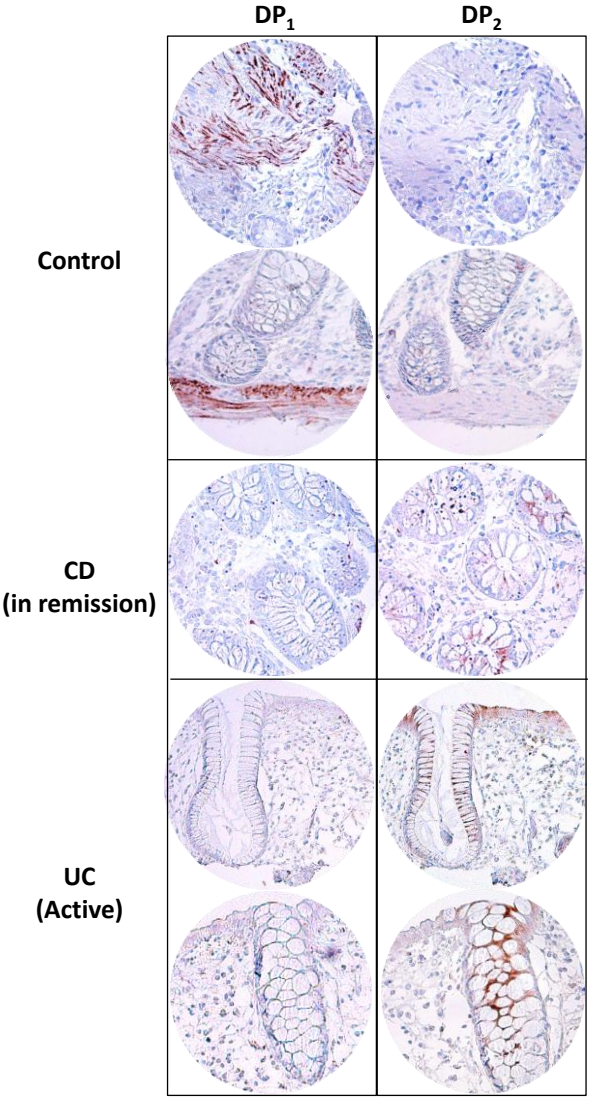


Figure 44. *Expression of prostaglandin D_2 receptors DP1 and DP2 in colonic biopsies from a healthy (a), an ulcerative colitis (b) and Crohn´s disease in remission patient. . Immunohistochemistry was performed.*

To gain a better understanding of the system of DP_1/DP_2 expression pattern, and the modulation of their expression by proinflammatory cytokines, we studied the protein expression of both PGD_2 receptors in Caco-2 cells by Western blot, incubating the monolayer 24 hours with proinflammatory cytokines (IL-1β, TNFα, IFNγ). As Figure 45 shows, the cytokines could modulate the expression of both receptors but in opposite directions. DP_1 is upregulated by IL-1β, and reduced by TNFα or IFNγ. On the other hand, DP_2 is only upregulated by TNFα or IFNγ. However, these are preliminary results and a big set of experiments is needed to get a conclusion.

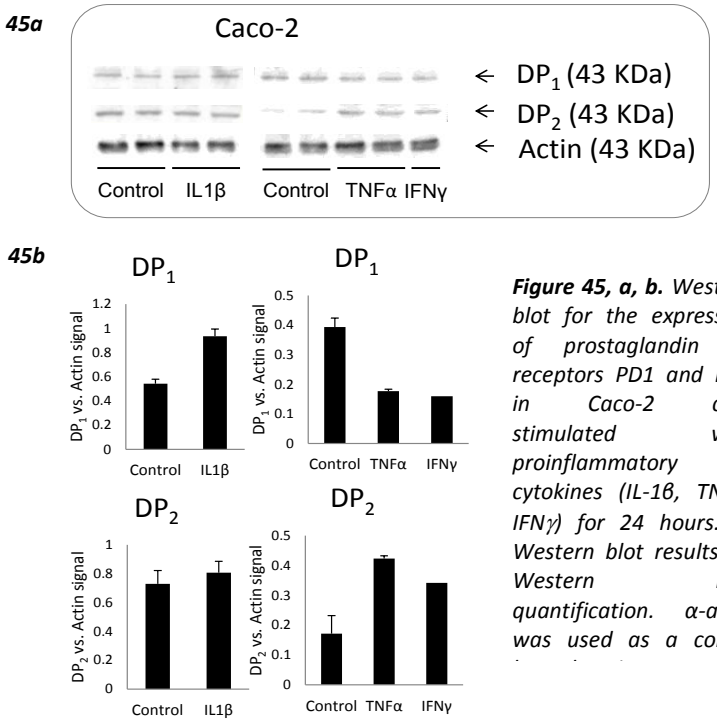


Figure 45, a, b. *Western blot for the expression of prostaglandin D_2 receptors PD1 and PD2 in Caco-2 cells stimulated with proinflammatory cytokines (IL-1β, TNFα, IFNγ) for 24 hours. a. Western blot results. b. Western blot quantification. α-actin was used as a contol*

- **Is the L-PGD shyntase expression modified in inflamed conditions in humans?**

  The same isoform studied in rats was studied by IHC in distal colon biopsies from healthy, CD and UC patients. As observed in the rat, *Figure 46* demonstrates *there is an increase in the expression of L-PTGDS in the IBD tissue versus the control patients.* Furthermore, CD4 was colocalized with PTGDS in IBD specimens (figure 47), suggesting that this enzyme is expressed by intraepithelial lymphocytes in inflammatory conditions.

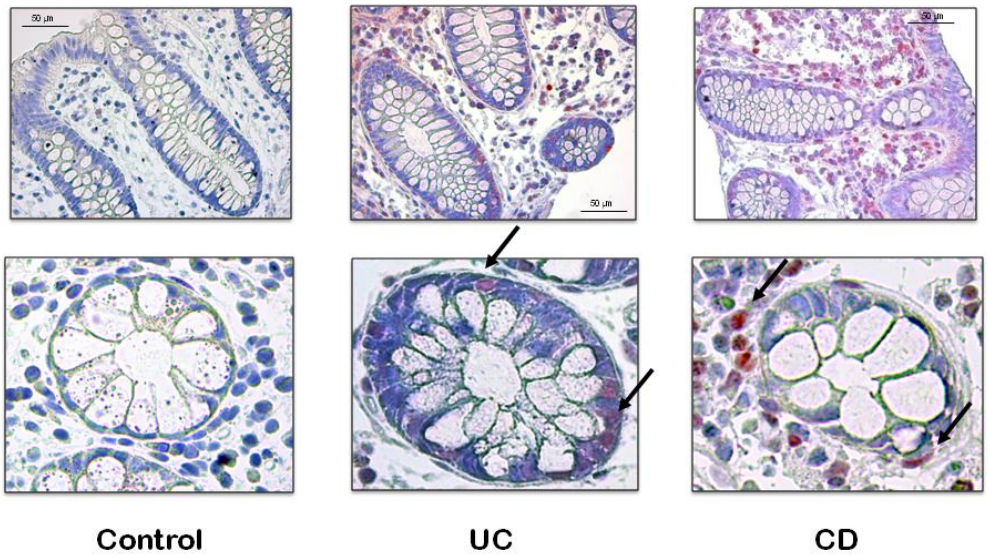


Figure 46. *Expression of prostaglandin D_2 synthase (L-PTGDS) in biopsies from healty (control), ulcerative colitis (UC) and Chron's disease (CD) patients. Immunohistochemistry was performed.*

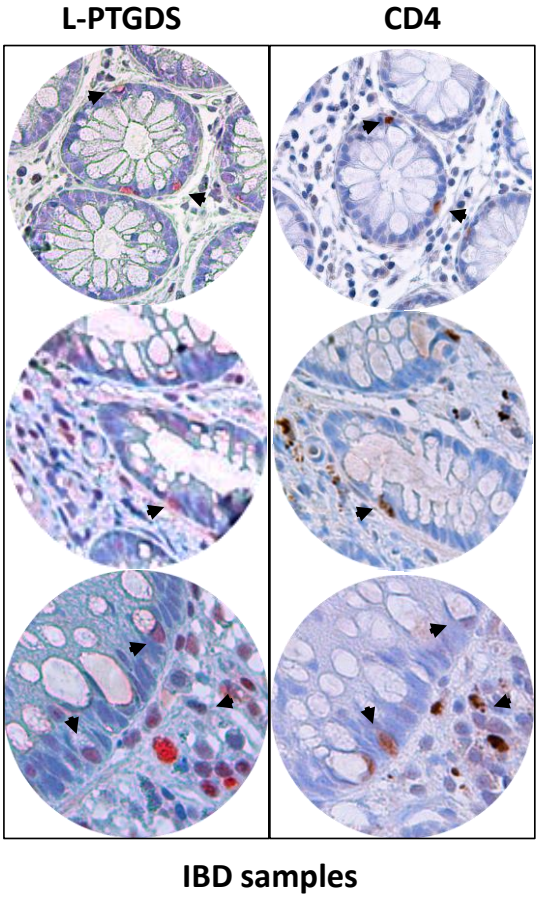


Figure 47. *Colocalization of L-prostaglandin D_2 synthase (L-PTGDS) and CD4 in biopsies from IBD specimens. Immunohistochemistry was performed.*

5. Can G_i protein stimulation account for the enhancement of carbachol induced secretory response?

So far we have shown similar results obtained with two mediators that share an activation of epithelial G_i proteins as part of their mechanism of action, namely UFP-512 and PGD_2. Both therefore are expected to decrease epithelial cAMP levels in response to receptor ligation (δ opioid and DP_2 receptors, respectively). In both cases we have been observed the same behavior:

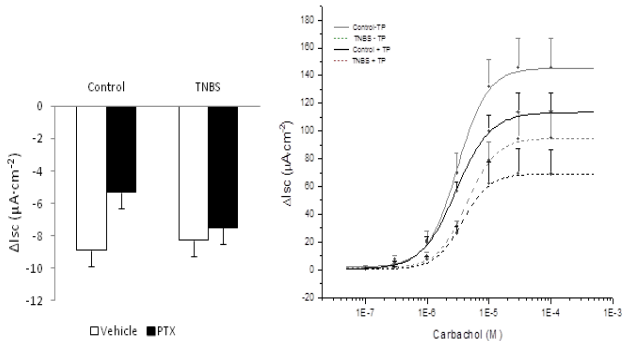


Figure 48 a, b. Effect of Pertussis Toxin (PTX), a Gi inhibitor, in rat colon of mucosa-submucosa preparations from the colon of colitic and control rats. Colitis was induced by the administration of TNBS. PTX (200 ng/ml) was added to the apical side for 20 min Isc was meassured in Ussing chambers. Basal secretion values (a), and concentration/response curve to carbachol(b) are shown. n=10-13. Data are expressed as mean ± SEM.

A third well known G_i protein modulator is Pertussis Toxin (PTX), which induces the ADP-ribosylation of G_i, resulting in inhibition of its function. We preincubated normal and inflamed colonic preparations in Ussing chambers with PTX (200 ng/ml apical) for 20 min and then obtained concentration response curves to carbachol (*Figure 48*). PTX had no effect on basal secretion. However, the addition of PTX to the inflamed rat colon mounted in Ussing chambers lowered both basal

- PGD_2 (1-16 μM) produced a sustained decrease in basal Isc in the rat colon, consistent with the activation of DP_2 receptors. Conversely, this prostanoid increased the maximal secretory response to carbachol, in a concentration dependent fashion (*Figure* 37).
- The δ opioid receptor agonist UFP-512 (1-10 μM), which also activates G_i protein, had similar effects on the Isc of noncolitic rats, so it had antisecretory effects in the basal secretion, and prosecretory effects in the carbachol curve (*Figure 29*).

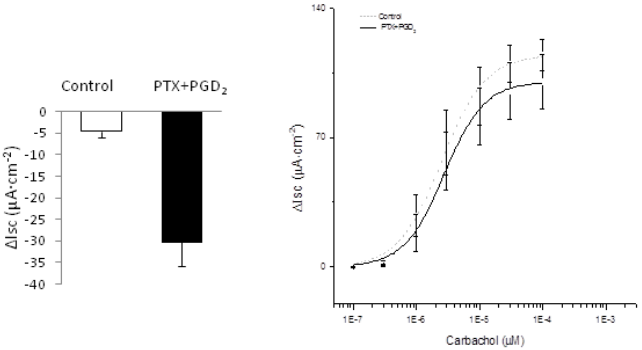


Figure 49 a, b. Effect of Pertussis Toxin (PTX), a Gi inhibitor, and prostagalndin D_2 (PGD_2), in rat colon of mucosa-submucosa preparations from the colon of control rats. PTX (100 ng/ml) was added to the apical side for 15 min, then PGD_2 (16 μM) was added for another 15 min. Isc was meassured in Ussing chambers. Basal secretion values (a), and concentration/response curve to carbachol(b) are shown. n=8-9. Data are expressed as mean ± SEM.

and carbachol evoked secretory response. A similar degree of inhibition was observed in normal and inflamed tissue.

In addition, we preincubated normal colonic preparations in Ussing chambers with PTX (15 min, 100 ng/ml), and then treated them with PGD_2, (16 μM, 15 min). This resulted in downregulation of basal secretion, comparable to that obtained without PTX (*Figure 49*). However, the enhancing effect on carbachol evoked response was no longer noted after PTX pretreatment.

These results prompted us to formulate an hypothesis based on the known ability of G_i proteins to activate K^+ channels [577] (see Discussion).

Our results globally suggest that, in addition to inhibition of cAMP generation via downregulation of adenylate cyclase, Gi proteins may paradoxically potentiate carbachol stimulated secretory responses by an unidentified mechanism that could be related to activation of K^+ channels.

6. Is PGD_2 a relevant player in TNBS colitis? Inflammatory response and intestinal transport

While H-PGDS is proinflammatory in allergic airway diseases, it has been shown to be protective in other models of inflammation. To evaluate role of H-PGDS in colitis, we inhibited H-PGDS and blocked DP_2 receptor administering HQL-79 (1mg/Kg) and ramatoban (10 mg/kg), respectively, in the TNBS model of rat colitis.

- HQL-79, a selective inhibitor of H-PTGD, is a synthetic tetrazole compound originally prepared as a possible antihistamine. It therapeutic effect when used in animal models of allergic disease and neuroinflammation. Evaluation in models of allergy and asthma demonstrated that HQL-79 is orally available, and that it inhibits the synthesis of PGD_2.
- As mentioned above, ramatroban is a DP_2 antagonist (IC_{50} values of 100-170 nM), which also blocks thromboxane receptors. It is actually an approved human medication for the treatment of allergic rhinitis. Ramatroban is more potent at the DP_2 receptor than the TP receptor by 4-5 fold.

The colonic inflammatory process induced by TNBS was associated with a decrease in rat body weight when compared to control rats, most probably due to anorexia and the presence of diarrhea in the colitic animals. Non significant differences were found among colitis groups in body weight (*Figure 50*). Food intake was significantly lowered in all colitic groups

compared to the control, but there was no effect of HQL-79 or ramatoban. No changes in the water consumption were either observed (dada not shown).

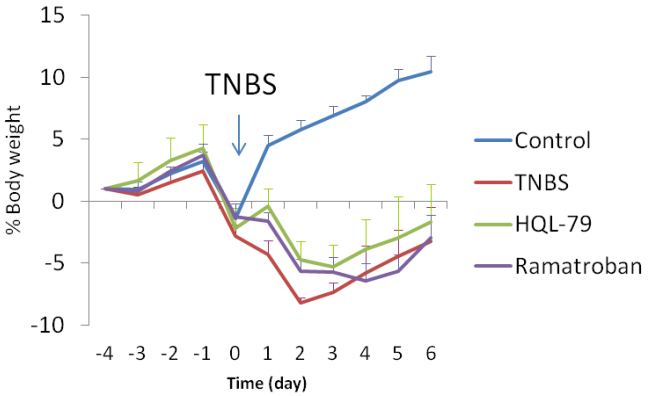


Figure 50. *Body weight gain in rats treated with HQL-79 and ramatroban. TNBS colitis was associated with body weight loss. n=6.*

Macroscopic and microscopic analysis of the colonic samples was performed. Colonic weight to length ratio was measured, with an increase in all the colitic groups related to an enhancement of weight and a reduction in length (*Figure 51*). The damage score was also increased by the administration of TNBS. No changes among colitic groups were observed. However, it should be noted that HQL-79 and ramatroban reduced the hyperemia and obstruction score, while HQL-79 also decreased deformation.

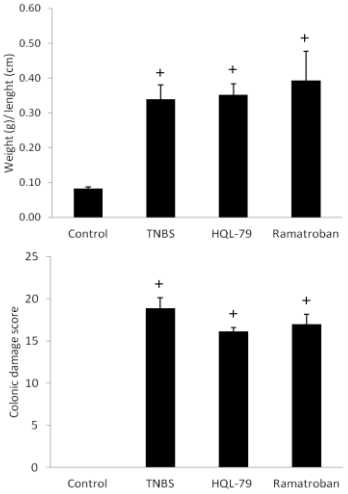


Figure 51 .Colonic weight (a), damage score (b) in rats treated with HQL-79 and ramatroban. Animal status was examined at 6 d postcolitis. TNBS colitis increased all two parameters. n=6. + 0.05< vs. Control group.

Microscopically, colonic samples colonic samples from the TNBS control group showed severe transmural disruption of the normal architecture of the colon, characterized by extensive ulceration and inflammation involving all the intestinal

layers (*Figure 52*). The inflammatory process was also associated with crypt hyperplasia and dilatation, and moderate to severe goblet cell depletion. In contrast, histological analysis of the colonic specimens from rats treated with both HLQ-79 and ramatroban, specially with the first one, presented a pronounced recovery with a significantly reduced score when compared to untreated rats. The transmural involvement of the lesions was reduced and most of the samples showed a restoration of the epithelial cell layer with crypt hyperplasia scarcely observed. In addition, there was a replenishment of goblet cells in which a higher mucin content could be observed.

The biochemical analysis revealed that HQL-79 and ramatroban significantly ameliorated the colonic MPO activity, a marker of neutrophil infiltration that was enhanced in the TNBS group (*Figure 53*). However ramatroban was also able to reduce this enzyme activity and expression, although it showed no significant anti-inflammatory effect when macroscopically evaluated.

Colonic expression of inflammatory cytokines was studied by RT-PCR (*Figure 57*). The results show that the animals in the TNBS group exhibit an increased expression of S100A8 (a neutrophil marker, which together with S100A9 forms the calprotectin dimer),TNFα and IL-1β, although the high variability prevented this difference from reaching statistical significance. HQL-79 and ramatroban exerted an inhibitory effect on S100A8, IL-1β, INOS, MCP-1 or TNF expression that was similar for both inhibitors (*Figure x*).

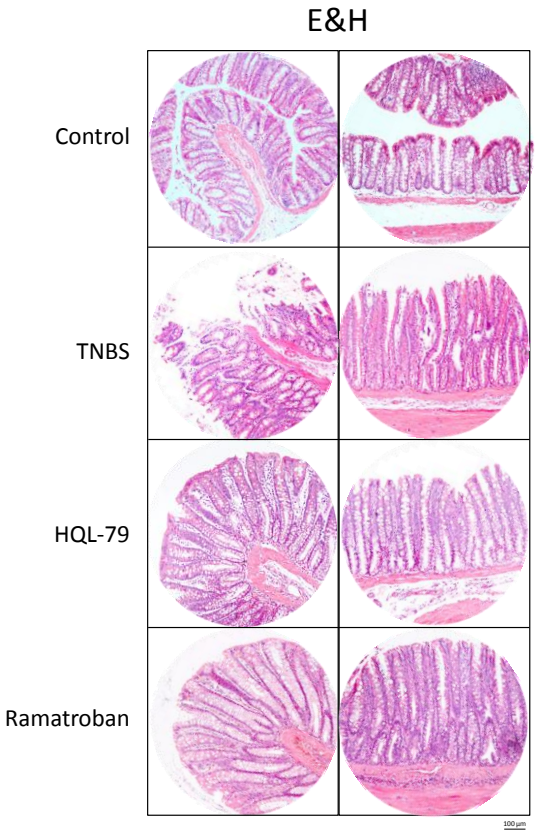


Figure 52

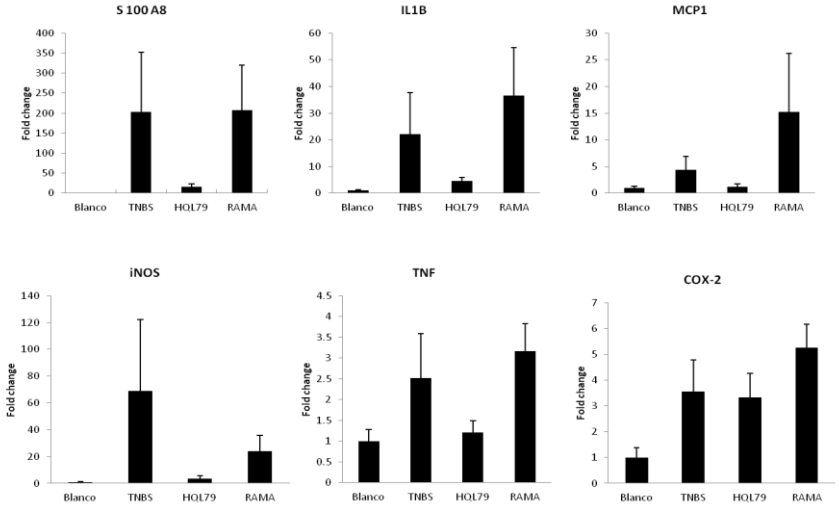
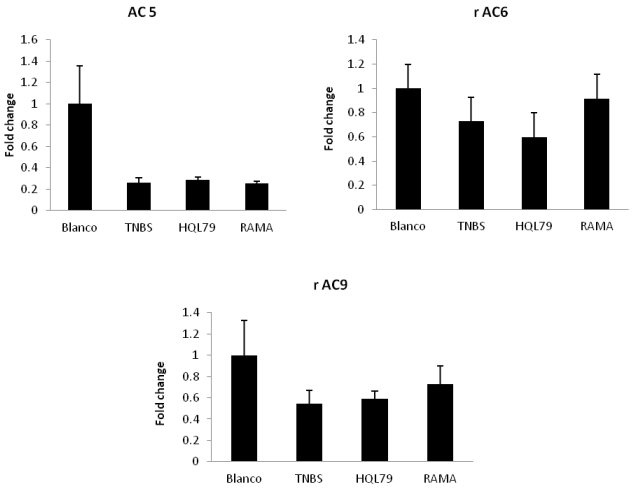
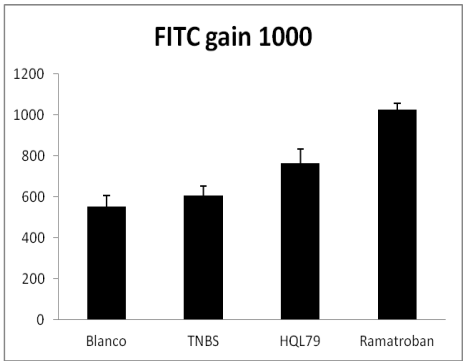


Figure 53 and 54

We further studied the expression of different isoforms of AC. As shown in *figure 54*, and in agreement with previous results (see part I) the expression of AC5, 6 and 9 was decreased by the administration of TNBS. Neither HQL-79 nor ramatroban had any effect on the expression of these AC isoforms.



The effect on the ion secretion of all groups was studied with Ussing chambers. Despite the positive, albeit limited, effect on inflammation, there was no effect on transport. As *Figure 55* a shows that the effects of inflammation on stimulated carbachol receptors could not be reverted by any of the inhibitors. A permeability assay with FITC-D was also performed showing that HQL-79 and ramatroban increased the FITC-D permeability (*Figure 56*).



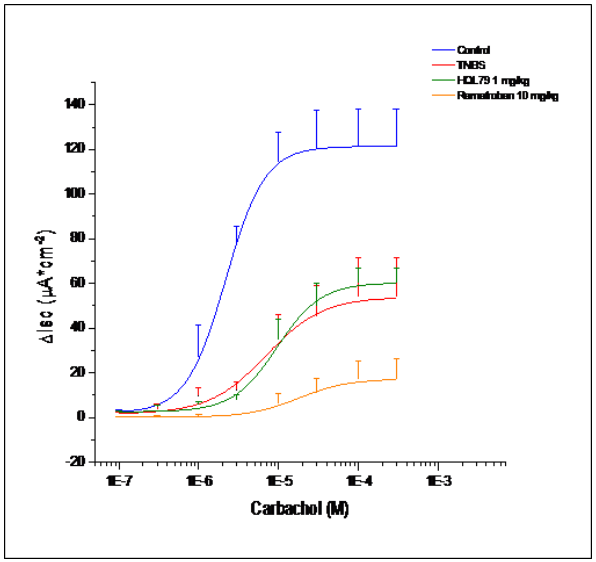


Figure 55 and 56*. Ussing chambers Isc and FITC-D permeability.*

PART III

Reversible Ponceau staining as a loading control alternative to actin in Western blots

Gels and blots are one of the fundamental tools of molecular biology. Their foremost utility is to assess the characteristics of nucleic acids and proteins in electrophoretic movement, indicating changes in size, glycosylation, dimerization and so on. However, they are also used frequently to measure quantitative changes in gene expression among samples, in spite of the known limitations of this approach, including a small dynamic range and a certain unpredictability. In particular, the quantitative use of Northern blots has been classically hampered by the characteristic liability of RNA, because degradation can easily pass for downregulation in the blot. Hence parallel measurement of a housekeeping gene, such as actin, 18 S or glyceraldehyde-3-phosphate dehydrogenase (GADPH), has become commonplace. This procedure is known as 'loading control', but this is probably a misnomer since its primary objective is to rule out degradation, not faulty gel loading, as a confounding factor; since pipetting is required in virtually every experiment, this line of reasoning would lead to the use of 'loading controls' for each one of them.

At any rate, the 'loading control' practice is increasingly extending to Western blots, and it is required by many journals, even though the need for this step is debatable. Ponceau S staining of proteins has long been applied to quality control of membrane transfer in Western blotting. Ponceau S staining is fast, unexpensive, nontoxic and, above all, binding is fully reversible in a few minutes. Here we propose to use Ponceau S staining as an alternative means to actin immunoblotting to assess equal loading in Western blots.

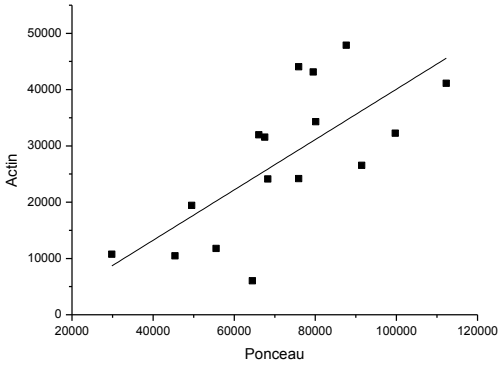


Figure 57. *Correlation between Ponceau S and actin densitometric signal. Rat colon, kidney and liver samples were analyzed by Western blot, including reversible Ponceau S staining prior to incubation with primary antibody.*

In order to do this, we obtained samples from different organs derived from two female Wistar rats of approximately 200 g of weight. The animals were sacrificed by cervical dislocation and colon, liver and kidney samples were extracted, homogenized in RIPA buffer with freshly added protease inhibitors (phenyl-methylsulfonyl fluoride, aprotinin, leupeptin, pepstatin A, 1,10-phenanthroline), cleared by centrifugation, and heated at 95°C for 4 minutes in denaturing Laemmli buffer. The composition of the RIPA buffer is: 0.1% sodium dodecylsulfate, 0.1% sodium deoxycholate, 1% Triton X-100 in phosphate buffered saline. The composition of the Laemmli buffer (5X) was: 312 nM SDS, 50% v/v glycerol, 1% v/v 2-mercaptoethanol, 22.5 mM EDTA trisodium salt, 220 mM Tris and traces of bromphenol blue (pH=6.8). Protein content was measured by the bicinchoninic acid method, using bovine serum albumin as standard [578] . Then we ran a series of Western blots with varying loading conditions and assessed gel loading either by Ponceau S staining (right after transfer) or by actin immunoblotting using the JLA-20 monoclonal antibody. The JLA20 antibody against actin developed by Dr. Lin [579] was obtained from the Development Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA). The secondary peroxidase conjugated antibody was from Sigma (Barcelona, Spain). We used Biorad electrophoresis and transfer units and Schering-Plough nitrocellulose membranes, with 90 min transfer time and 100 V constant voltage settings. After transfer, the nitrocellulose membranes were rinsed briefly in distilled water and incubated in Ponceau S solution (0.5 w/v in 1% v/v acetic acid) for 2 minutes [580] , followed by a brief rinse in distilled water, so that the lanes and bands are clearly visible. The membranes were then inserted in between transparency sheets and scanned at 300 dpi to a TIFF file using a standard scanner (EpsonPerfection 3490 Photo). After that the membranes were rinsed once more in distilled water for 2-3 minutes, until the staining was completely eliminated, and we proceeded with the blocking and antibody incubation steps, as usual. Antibody bound peroxidase was detected by enhanced chemoluminiscence (Perkin Elmer™, Life Sciences, Boston, USA), documented in Kodak film and quantitated with Scion Image software. Because Ponceau S is a nonspecific protein dye, all proteins in the membrane are colored. We selected a section of the blot that covered a wide range of molecular weights in each case, typically ~90% or more of the lane length.

We initially tested the capacity of reversible Ponceau S staining to assess equal gel loading, and accordingly ran a series of gels featuring the different tissue samples at equal levels of protein loading, namely at 140, 80, 40 and 10 µg of protein per lane, thus covering the entire range of ‘small’ gels. *Figure 1*

shows a typical Ponceau stain and actin signal. Clearly, the results are very similar with both methods. As expected, there are no differences depending on the organ considered (not shown). However, the slopes do differ depending on the organ, so that correlation for each individual type of sample is typically around 0.9 (r^2), while linearity is impaired in the graph showing the combined data.

As alluded to before, Western blots may be used for quantitative purposes, although this approach has well acknowledged limitations, particularly in terms of the dynamic range, i.e. the difference between the threshold signal that is detectable and the saturation point, beyond which the signal is not further increased or it is increased only marginally even though the protein detected may be present in much higher amounts. Thus we set out to verify whether the dynamic range is different for Ponceau S and actin immunoblotting. The analysis of the densitometric quantitation of the Ponceau S and actin signals is shown in *Figure 2*. The results are reasonably linear up to 140 µg of protein loaded in both cases, indicating that there is no limitation of dynamic range imposed by the use of Ponceau S compared to actin. Actually, the linearity was enhanced with Ponceau S in this particular experiment, as indicated by the higher difference between the maximal and minimal densitometric values. It should be noted however that Western blotting

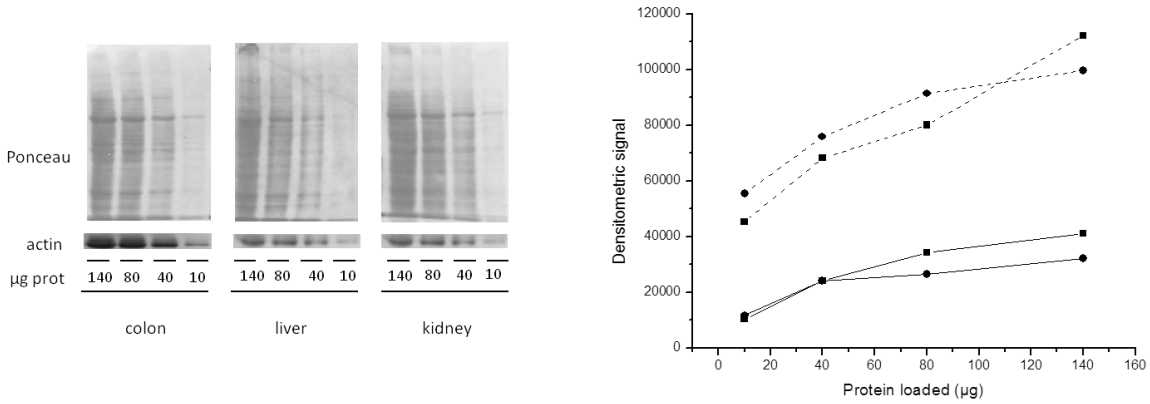


Figure 58. *Dynamic range of actin and Ponceau S densitometric signal. Rat kidney (circles) and liver (squares) samples in different quantities (10-140 µg) were analyzed by Western blot, including reversible Ponceau S staining prior to incubation with primary antibody. Both actin (solid lines) and Ponceau S (broken lines) densitometric signals are shown.*

has well known limitations for quantitative purposes either way.

Our data provide validation for the use of reversible Ponceau S staining to assess equal gel loading, or quality control, in Western blots. Certainly, there are limitations to our validation. In principle it applies only to the conditions used, i.e. rat colon, kidney and liver. We have studied also mouse organs as well as HT29, T84 and Caco-2 human cells, with similar results. Moreover, our method can be extended to any other type of sample with minimal effort. For instance, it seems to work equally well for bone and jejunum rat samples (although this was not tested extensively). The same applies to technical conditions in our study, such as the use of nitrocellulose membranes, blocking buffer, antibodies and so forth.

Ponceau staining has an additional advantage: it does not rely on a single protein for normalization or loading control. This circumvents the possibility that the 'housekeeping' proteins used for this purpose may actually vary in some conditions, or that they are saturated at the levels of loading necessary for detection of low expression products [581, 582].

In conclusion, we have shown that reversible Ponceau S staining can be used advantageously over actin detection for quality or equal loading control in Western blotting. It is equally useful for this purpose, has similar or improved dynamic range, is extremely inexpensive, and takes no longer than 10 minutes.

DISCUSSION

As explained in the Aims of this thesis, the lack of substantial modulation of transport/barrier funcion by the nutraceutical agents with intestinal antiinflammatory activity which were originally proposed to be studied moved us to change the focus of the research toward an in depth characterization of the related pathophysiology of IBD itself. Thus the work presented in this doctoral thesis represents a continuation of previous efforts of our research group which were published a few years ago [26, 479], along with those of other laboratories led by Dr. Wallace MacNaughton, Dr. Ursula Seidler or Dr. MacKay, among others, which have focused on the disturbances of ion transport in IBD. The current scenario that can be drawn from this set of studies has been reviewed by us [43, 44] can be summarized as follows:

- As opposed to acute colitis, which is typically accompanied by enhanced hydroelectrolytic secretion, chronic colitis in humans and animal models is characterized by inhibited absorption and secretion.
- These disturbances in ion transport have been linked in part to changes in the epithelial transportome, brought about by proinflammatory cytokines such as IFNγ or TNFα, but also to regulatory actions attributed to nitric oxide, PGD_2, changes in the cholinergic nervous system within the gut, or deficit in epithelial cAMP levels.
- Inhibited ion secretion (Isc in Ussing chambers) is at least partly reversible at short term in vitro, suggesting that regulatory modulation of transport is involved in this phenomenon.

Our own results pointed to a low epithelial cAMP level as a key element in diminished ion secretion. This was derived mainly from the analysis of concentration response curved to Ca^{2+}- and cAMP- dependent secretagogues, but direct measurement of cAMP in primary enterocyte preparations confirmed this fact [26]. We also observed that the defect in ion secretion was largely reversed in mucosal preparations, as opposed to the more standard mucosa-submucosa preparations, suggesting an important regulatory role of the submucosa. However, in this new set of experiments we could only achieve a partial reversal. This discrepancy was not due to an overrepresentation of 'successful' experiments in our previous study, because in both cases the experimental group size was large (n=20). Thus we attribute it to happenstance. Given the nature of the epithelial transport defect in chronic colitis, it is indeed no surprise to obtain partial rather than total reversal by any experimental manouver.

We followed various lines of research in this project more or less simultaneously, although for clarity they are presented separately and in an orderly manner in this thesis. Basically, we were interested in assessing whether changes in the transportome could underlie the transport defect, and we naturally were keen on exploring the origin of

low epithelial cAMP. Both ends could be partly addressed by carrying out an analysis of the transcriptome of epithelial cells, since this features both transporters and regulatory genes, along with virtually the entire rat gene set. To this end we used microarray analysis, namely the same technological platform applied formerly by our group [557, 558]. The Rat Expression Array 230 2.0 (Affymetrix®) microarray features ~30.000 genes and therefore the vast majority of the rat genome, although there are a few exceptions (e.g. myeloperoxidase) and there are quite a few nonanotated sequences, which is an expected consequence of the reduced knowledge of rat genes compared to human and mouse counterparts. Based on our previous efforts, and considering the current status of microarray derived genomic analysis, we decided to use this experiment solely as an hypothesis generating tool, and thus we pooled epithelial samples obtained from 6 different rats for a single microarray in each group (i.e. control and colitis). The main reason is that while obviously experimental error is reduced by using one microarray per sample, this has little practical importance because independent analysis is always required. We also focused on biological implications of the data, commonly referred to as functional genomics, rather than on extensive postgenomic validation by RT-qPCR. Hence we carried out validation on only a few genes and built on microarray and related data to extend and confirm our working hypothesis.

We used the same IBD model here, rat TNBS colitis, as well as in the remainder experiments featured in this project, that we did in our previous reports, for internal consistency. It should be noted that, although not characterized as extensively, we have confirmed that the basic ion transport defect is observed in several other models of IBD [25], in addition to human samples. Thus the mechanism operative in rat TNBS colitis are expected to be the same as, or closely related to, the ones in human IBD. For the same reason we used carbachol as prototype secretagogue in transport experiments discussed below, focusing on global transport phenotype assessed by a sensitive and rapid acting agent.

Our precedent genomic database using the rat TNBS model also allowed us to compare the changes in the transcriptome observed in the enterocyte preparation with those in full thickness tissue. As expected, there was some leakage of nonepithelial genes into the epithelial samples, since these are not pure preparations. The magnitude of upregulation of inflammatory genes were markedly lower than in full tissue, consistent with enrichment of epithelial cells. Thus observations pertaining non epithelial specific genes (e.g. Atp1a1) should be taken with caution.

Our data did not show any changes in the transportome that could explain the defect in ion secretion or absorption, since key transporter genes were noted to be either unchanged or upregulated. This is in disagreement with some previous reports [38, 379, 510, 525](reviewed in [44]), but on the other hand consistent with some other studies. Although the lack of downregulation of key transportome players fits in well with the transport phenotype in this model, we did not pursue this goal further. However, since mRNA levels are not always directly correlated with protein levels, we did try to assess protein expression by Western blot. We must humbly acknowledge that our results here are not of good quality, even though a variety of antibodies were assayed. At any rate, we found no evidence of substantial changes here either. Therefore, our results suggest that the transport defect is related chiefly to regulatory changes,

which could involve also modulation in vesicle trafficking, a well known mechanism of regulation of transporters such as NHE3 or NKCC1 [583-585], whereby activity is altered by selective insertion or removal of the transporter into/from the plasma membrane of epithelial cells, with recycling endosomes acting as functional reservoir. Since overall mRNA/protein is unaffected, our microarray and Western blot analyses would not pick up such a mechanism. However, it is subject to well established regulatory pathways including cAMP.

The microarray did reveal changes in the mRNA levels of genes encoding proteins that serve transporter regulatory functions, plus genes involved in the cAMP pathway. For instance, the expression of PDZK1, the main regulator of NHE3 activity but also CFTR and DRA modulator [105], was found to be downregulated. This protein acts as an AKAP, or protein kinase A anchor protein, working as a bridge that leads PKA to NHE3 in the adequate membrane domains and ultimately permits NHE3 phosphorylation and the subsequent inhibition of NHE3 function [105, 106]. Of note, such alteration in PDZK1 expression without accompanying changes in NHE3 expression itself has also been observer by Ursula Seidler's group [559, 560] in the mouse lymphocyte transfer model of colitis and in human IBD patients. Similar results have been reported by Lenzen et al. in IL-10 knockout mice [524]. On the other hand, the microarray showed upregulation of PDZK3, which perhaps is a compensatory adaptation, although we do not have any functional data to interpret this. At any rate, it is interesting to note that a lower expression of PDZK1 would lead to reduced inhibition of ion absorption in the inflamed intestine, and this would tend to counteract diarrhea, suggesting an adaptive uncoupling mechanism.

With regard to regulation of cAMP, we observed changes in the expression of several phosphodiesterase isoforms. It is important to remark that PDE4C, the only PDE cAMP-specific and the major cAMP metabolizing enzyme found in inflammatory and immune cells, was upregulated in colonocytes from TNBS colitic rats. Because of the sheer complexity of the phosphodiesterase super family, which features at least 11 families [586], it is impossible to anticipate what is the outcome of this particular mixture of modulatory changes in expression. We focused instead on the adenylate cyclase system, which is simpler but still features no less than 10 isozymes [172]. Of these, three were expressed by normal colonocytes, 'colitic' colonocytes, or both: AC6 (Adcy6), AC9 and AC10. AC9 was significantly decreased in the colitic rats, while AC10 (soluble AC) was induced, considering that it was detected only in inflammation samples. In contrast, AC6 was detected in comparable levels in control and colitic samples. We applied postgenomic analysis to this set of genes, since they were of primary importance for us, using a separate set of samples analyzed by RT-qPCR. The results obtained confirmed the repression of AC9 and the epithelial expression of AC6. However, it further indicated the presence of AC5, although in very low amounts, and showed that both AC5 and AC6 expression was downregulated in TNBS colitis. This is consistent with the higher sensitivity and precision of the qPCR technique [558]. The reason why AC5 was measured in this experiment is that AC6 and AC5 are the two G_i sensitive adenylate cyclase isoforms, and therefore are obvious candidates for regulatory changes in cAMP in chronic colitis. Our data indicate also that the

predominant isoform in epithelial cells in both mice and rats is AC6. In fact, although the microarray showed inhibition of AC9 but not AC6 in the inflamed enterocytes, RT-qPCR indicates that AC6 is more affected than AC9. In contrast, in full thickness tissue AC5 is the predominant isoform, followed by AC9 and then AC6. These results are further evidence of the epithelial cell (relative) purity of our samples.

It is interesting to note that we observed very similar changes in AC expression in the colon of CFTR knockout mice. We measured adenylate cyclase expression in these samples because we had access to this well known model of cystic fibrosis, which is characterized by inflammation of the large intestine as part of the overall pathology. Thus this is confirmatory evidence of modulation of AC isoforms in the chronically inflamed colon in a separate, unrelated animal model, which like TNBS rat colitis and human IBD is characterized by decreased secretion of chloride and bicarbonate (although for different reasons).

Naturally, the inhibited expression of adenylate cyclase isoforms is consistent with a lower cAMP production and intracellular levels, which as noted is central to our hypothesis. However, the data discussed so far do not prove by themselves that there is a causal connection between these events. Therefore we set out to develop a functional validations of our findings. First, we identified the inflammatory cytokines TNFα, IL-1β and IFNγ as possible causatives of the downregulation of AC isoforms. Because primary epithelial cells show extremely low viability ex vivo, we assessed the short term effect of these cytokines in Caco-2 cells. Our results are fairly consistent with those observed in vivo. Because Caco-2 cells are of tumoral origin, we further validated our observations using mouse jejunal organoids. This type of stem cell derived structures are strictly non tumoral, non transformed, and are considered the best approach to epithelial biology research in vitro, although it is both expensive and technically demanding. We were fortunate to have the opportunity to work with Hugo de Jonge's group to learn the technique and to perform specific experiments in situ. In this case we were unable to study colonic specimens because unfortunately, for reasons unknown our colonic organoids do not survive in vitro. Our results, obtained in jejunal organoids, showed again an inhibitory effect of all three cytokines on AC5, AC6 and AC9 expression. We were close to measured the functional activity of these jejunum organoids [541] pretreated with the proinflammatory cytokines, however, the international fellowship was too short to carry out these experiments. Because TNFα, IFNγ and IL-1β are pivotal in IBD and experimental colitis models including rat TNBS colitis, our data strongly suggest that this modulation is a common and significant finding in chronic colitis.

Although not addressed by us, these cytokines, particularly TNFα and IFNγ, have been reported to exert modulatory effects on ion transport and specific transporters, notably Na^+/K^+ ATPase [12, 386, 514, 538, 539, 587]. These actions are physiologically consistent. Our own results confirm the overall inhibition of transport by IFNγ in T84 cells [386, 565, 588].

Next we addressed the functional consequences of AC downregulation. We focused on AC5 and AC6 because of their aforementioned G_i sensitivity and foreseeable relevance in the inflamed intestine. Unfortunately, for incidental technical reasons we have no funcional data on AC6 at this time, although the experiments are scheduled and will be performed before publication of these data in article form. Despite this obstacle, we obtained very reassuring data with AC5. T84 cells were used as model epithelial cell line to evaluate the effects of AC5 gene silencing. In these conditions we observed a significant reduction of both basal and carbachol- or forskolin-stimulated ion secretion as assessed by changes in Isc in Ussing chambers.

These data indicate that, despite its relatively minor expression compared to AC6 or AC9, the reduction of AC5 is enough to impide ion secretion substantially in T84 cells. This intervention is expected to decrease intracellular cAMP levels, thereby interferring with cAMP stimulated events including NKCC1 activation and membrane insertion, activation of basolateral K^+ channels, increasing gating of CFTR and inihiting NaCl electroneutral absporotion by interaction of PKA with NHE3 requiring auxiliary proteins. Because CFTR is the main common exit pathway for chloride and bicarbonate for a variety of secretory stimuli, an inhibition of basal and secretagogue dependent secretion is expected.

We also assessed the effect of adenylate cyclase inhibition in vivo, using the specific agent Ddado in mucosa-submucosa preparations of uninflamed rat colon mounted in Ussing chambers. Inasmuch as Ddado achieves an inhibition of AC activity, resulting in diminished cAMP levels, a similar reduction of Isc responses is predicted. Our data indicate that this manouver results in a decreased basal Isc, reflecting a lower secretion of chloride/bicarbonate. In the rat colon the response to carbachol is partly direct, mediated by the stimulation of basolateral M_3 muscarinic receptors in epithelial cells, and partly indirect, derived from the interaction of carbachol with nicotinic and/or muscarinic receptors elsewhere, at sites including the enteric nervous system, the submucosa and a variety of subepithelial cells. This indirect mechanism involves the release of mediators such as prostaglandins, that add to the secretory effects. In fact, prostaglandins are considered essential to the secretory effect of carbachol and other Ca^{2+} dependent stimuli because they cannot stimulate CFTR opening by themselves. Instead, they rely on the constitutive and induced production of cAMP operating mediators that serve this permissive role [26, 30, 135, 480]. Basal Isc in the rat colon is inhibited by tetrodotoxin, atropine and indomethacin [589], indicating that it is dependent on activation of muscarinic receptors, prostaglandin release, and input from the enteric nervous system. Thus the fact that Ddado inhibits spontaneous Isc tone is consistent with interference with the dependence from a basal level of cAMP stimulation. Because Caco-2 cells lack this kind of spontaneous input from other cells, it makes sense that AC inhibition has a more powerful effect, shutting down overall transport more effectively. However, this does not explain why the inflamed rat colon displays marked inhibition not only of basal secretion, but also of the secretory response to carbachol, IBMX, histamine and other secretagogues [25]. Since these agents are studied at submaximal concentrations, it is likely that

they overcome the effects of AC inhibition by Ddado, while it results in substantial dampening of basal, low intensity secretion. Also, the fact that Ddado was added apically supports that the epithelium is maximally affected.

Taken together, our results are consistent with a role of lowered expression/activity of epithelial adenylate cyclase isoforms in the defect of ionic secretion observed in the chronically inflamed intestine.

As discussed above, inflammatory cytokines may downregulate adenylate cyclase expression in intestinal epithelial cells. An alternative mechanism is the inhibition of the AC5 and AC6 adenylate cyclase isoforms by way of stimulation of G_i proteins. This led us to explore some of the mediators capable of exerting such an action in the inflamed intestine. Some of the possibilities include Nitric oxide, endogenous opioids, somatostatin or prostaglandins. Nitric oxide is one of the molecules that has received most attention regarding alterations of ion transport in inflammation. Thus IBMX and electrically evoked secretion is partly reversed by in vitro incubation with the iNOS inhibitor L-NIL [28]. Although the possible mechanism of NO was not considered in those studies, it should be noted that NO has been reported to inhibit adenylate cyclase [30]. Thus an involvement of NO in ion transport defects is fairly consistent with our hypothesis, in principle. Bearing this in mind, the fact that carbachol induced secretion is not affected by iNOS blockade in experiments parallel to those in which IBMX and electrical (i.e. enteric nervous system) stimulation is partially reversed remains unexplained and hinders our mechanistic understanding. Furthermore, our extended experimental database has consistently failed to show any change in either basal or carbachol evoked secretion in the colon of TNBS colitic rats after L-NIL treatment. Given the positive results obtained by other groups however, we decided to explore this pathway further. Two additional experiments were carried out. In the first one an NO donor was assayed in uninflamed rat colon mounted in Ussing chambers, and the effects were unambigously prosecreteory, since both basal and carbachol evoked secretion were significantly enhanced compared to the control. Thus NO acts as a secretagogue in this tissue in normal conditions. This is in agreement with previous observations, and in fact NO has been claimed to contribute to the mechanism of action of clinically used laxative agents [590]. Obviously this result is incompatible with a role of NO in diminished ion secretion, except if its effects are altered in an inflammatory context. The second experiment was designed to address this possibility, and the destruction of tissue NO by a specific scavenger (as opposed to inhibited NO generation by L-NIL) was not shown to have a significant effect on basal or carbachol elicited Isc values. However, a trend toward a higher basal Isc was noted. It is possible therefore that a small effect may be picked up (i.e. become significant) using an expanded group size, although the one used in our study is substantial (n=8-14). Even considering this eventuality, our data do not support a major role of NO in the inflammatory defect in ion transport, but it remains possible that it is involved to a certain degree. In particular, our data are suggestive of a change in the predominant effect of NO on ion secretion. This in turn is hardly surprising because NO has been reported to have both prosecretory and antisecretory effects by a variety of experimental groups [30, 151].

A second type of mediator that could be involved by epithelial G_i activation is opioids. All opioid receptors are linked to G_i and the subsequent inhibition of adenylate cyclase. In fact, this action is of paramount pharmacological importance, since one of the main antidiarrhoeal agents, loperamide, acts by inhibiting ion transport and motility via occupation of μ and other opioid receptors [591]. Another antidiarrhoeal drug, racecadotril, is an esfingomyelinase inhibitor that indirectly promotes an enhancement of δ receptor mediated effects. This approach has the advantage of not affecting intestinal motility, since lack of appropppriate propulsive movements may result in toxic megacolon [592]. By using the δ receptor selective agonist UFP-512 (a kind gift of Prof. Gianfranco Baldoni,University of Cagliari, Italy), we obtained the expected reduction of basal Isc in uninflamed rat control specimens. Although the subsequent response to carbachol was not inhibited, this may be a consequence of insufficient adenylate cyclase blockade in the face of a maximally acting stimulus, as discussed above. However, we obtained no reversal of depressed transport in colitic tissue using either naloxone (a general opioid blocker with preference for μ receptors) or naltrindole (a δ receptor antagonist). Hence our data rule out a significant role of opioids in inhibited transport in the chronically inflamed intestine.

We then focused on prostaglandins, namely on PGD_2. Prostaglandins signal through 7 membrane spanning receptors coupled to G_s (PGE_2 EP_2 and EP_4 receptors, PGI_2 IP receptor and PGD_2 DP_1 receptor) proteins, which stimulate adenylate cyclase, or to $G_{q/11}$, which activated the Ca^{2+}/IP_3 pathway (TXA_2 TP receptor, EP_1 receptor and $PGF_{2\alpha}$ FP receptor). The PGD_2 DP_2 receptor and the PGE_2 EP_3 receptor in the other hand activate G_i proteins and therefore inhibit adenylate cyclase. A role of PGD_2 in defective secretory epithelial responses was suggested previously by Zamuner et al. [473].

Our results confirmed that PGE_2, PGI_2 and $PGF_{2\alpha}$ act as secretagogues in the normal rat colon, but this effect is largely exerted at the submucosal level rather than directly on epithelial cells. Conversely, PGD_2 has an antisecretory effect in standard mucosa-submucosa preparations. Although this was no longer observed in mucosal samples, this is still consistent with a direct epithelial cell effect mediated by DP_2 receptors, because mucosal preparations have a very low spontaneous ion transport and therefore further inhibition may be impossible to attain. This is further supported by the observation of decreased cAMP basal and induced levels in Caco-2 cells exposed to PGD_2 and their expression of the DP_2 receptor, which is also upregulated by TNFα.

In principle, because of the aforementioned permissive role of secretory prostaglandins in ion secretion in the rat colon, the defect in transport might be related to a reduced production of prostaglandins or to dampening of their effects. TNBS colitis in the rat has been associated with augmented production of PGE_2, [473, 593, 594]. Our data indicate that the response PGI_2 and $PGF_{2\alpha}$ is severely inhibited in the inflamed intestine, similar to the scenario with multiple other secretory stimuli, while that of PGE_2 is little inhibited. Since the secretory effect of these PGs is indirect however there is no clear connection with epithelial cAMP levels, and it is unlikely that there is a major involvement of

these eicosanoids in transport defects. The fact that PGE_2 maintains a solid response in the inflamed intestine is intriguing. Using concentration response curve analysis we showed previously that the inflamed mucosa was less sensitive to PGE_2, although maximal secretion was unaffected. Since expression of the EP_2 receptor was increased in TNBS colitic enterocytes, it is possible that enhanced receptor signaling may compensate partly the intrinsic defect in cAMP generation, according to our hypothesis. At any rate, PGE_2 also has no role in the defective ion secretion.

Our results show that blockade of DP_2 receptors with ramatroban in Ussing chambers partially reversed the defect in basal secretion, based on the significant increase in Isc observed in TNBS colitic rat colon. As expected, there was no effect of the DP_1 blocker AH6809. In their study, Zamuner et al. studied only alterations in transport in the late, postcolitic phase of rat TNBS colitis, i.e. when inflammation has actually resolved. Although they claimed that PGD_2 was involved, their data reflect similarly increased colonic PGD_2 levels at 1 day, 2 or 6 weeks after colitis induction, with the only exception of the determination at 1 week, which looks very much like an artifact. Hence there is hardly any reason to assume that the PGD_2 involvement is restricted to the postcolitic phase. In their study, PGD_2 (1 μM) inhibited basal Isc and the secretory response of the uninflamed rat colon to IBMX and electrical stimulation, while in our study it also reduced basal secretion but failed to inhibit the response to carbachol. This divergence points to a higher sensitivity of cAMP dependent secretion. Perhaps more importantly, Zamuner et al. rescued defective ion secretion in postcolitic rats with the COX2 inhibitor celecoxib, while we obtained comparable results with a DP_2 receptor blocker. It should be noted that indomethacin does not reverse inhibited ion transport in rat TNBS colitis [26], indicating that COX1 inhibition cancels the effect of interference with COX2 or a discrepancy of technical origin. At any rate, our results are consistent with upregulation of PGD_2 based on the increased expression of L-PTGDS. Scheduled, but untested, remain the measurement of the H isoform and of PGD_2 itself. The expression of the DP_2 receptor was unchanged in colitic rats. Of note, we observed a shift in the molecular weight of DP_1 in colitis, suggesting a postranslational modification, which warrants further investigation.

Taken together, we conclude that PGD_2 is involved in depressed ion secretion in rat TNBS colitis acting on DP_2 receptors. We then moved on to examine our hypothesis in human samples, through our collaboration with Ursula Seidler's laboratory. From a technical point of view we had the chance to study ion transport in Ussing chambers and perform histology/IHC studies with IBD and control samples. Contrary to our expectations, PGD_2 did not cause a decrease in basal Isc in human biopsies with no inflammation, but actually acted as a secretagogue in these conditions, reflecting important differences between the rat and human colon. However, ramatroban did show a trend toward increased basal and carbachol elicited secretion in samples from IBD patients. This did not reach statistical significance but the study was underpowered to appreciate such an effect, so that the data are in principle consistent with a role of PGD_2 in the human inflamed intestine. In fact, differences in PGD_2 receptor expression may account for the observed discrepancy between rats and humans. DP_1 was the only receptor expressed in the uninflamed intestine in humans, and it was detected at the submucosal rather than epithelial level. Conversely, IBD samples showed induction of DP_2 and

lower expression of DP_1, even in remission state. This result is in agreement with previous observations [471, 475]. Interestingly, L-PTGDS was shown to be expressed by intraepithelial lymphocytes in control and specially IBD specimens, suggesting that it can act on nearby epithelial cells in a paracrine fashion. We could not get reliable IHC results in rats, and therefore the cell pattern expression of DP_1, DP_2 or L-PTGDS is uncertain.

So far we have discussed most of the transport results but we have postponed some conflicting issue on purpose, to address them separately. Namely, PGD_2 was shown not only not to inhibit the concentration response curve to carbachol, but to actually enhance it. In order to confirm or discard this finding, we forced the conditions increasing the concentration of PGD_2, the result was a concentration dependent stimulatory effect. This effect is paradoxical because of the decrease of basal Isc observed in the same experiment. Notably, the exact same effect was observed with the δ opioid agonist UFP-512, i.e. reduction of basal Isc and increase of the response to carbachol. Because both agents act by activation of G_i proteins we tested the effect of pertussis toxin, an irreversible inhibitor of G_i proteins. In these conditions there was no change in basal Isc but the subsequent secretory response to carbachol was depressed in both control and colitic samples. We hypothesized that G_i proteins may exert a dual effect on epithelial cells: inhibition of sensitive adenylate cyclase isoforms (AC5 and AC6) on one hand, resulting in reduced cAMP levels and decreased activation of NKCC1, Na^+/K^+ ATPase, K^+ channels and CFTR and overall lower transport. On the other hand, we postulate that G_i may activate K^+ channels directly, as shown in other cell types [577]. Opening of K^+ channels increases the membrane electric potential, thereby increasing the energy pushing chloride and bicarbonate out of the cell. This would be detected as an increased maximal secretory effect of carbachol, provided that the K^+ conductances are complementary to those activated by muscarinic agonism itself. In this regard, the fact that the secretory response to cAMP was unchanged suggests that cAMP operated K^+ channels are targeted by G_i proteins. The divergent effect observed in basal and carbachol elicited ion secretion indicate that the cAMP action predominates in the former, while the putative K^+ channel effect is predominant under maximal or submaximal carbachol secretory conditions. Our hypothesis is confirmed in part by the fact that pertussis toxin abrogates the effect of PGD_2 on the carbachol response (this also points to G_i rather than G protein $\beta\gamma$ subunits as pivotal players, which can also activate K^+ channels such as K kir3). However it failed to affect the modulation of basal transport, suggesting that this may be less sensitive to the toxin, or that the interrelation between these two effects is more complex. At any rate, PGD_2 was shown to have the same carbachol enhancing effects in human samples, indicating that this phenomenon is not restricted to the rat. We have scheduled experiments using multiple photon microscopy (available in the School of Pharmacy building by our request) and specific K^+ fluorescent tracers, namely Asante potasium green AM.

Since PGD_2 is involved in the inflammation related alterations in colonic hydroelectrolytic transport, we decided to explore the possibility that long term interference with this pathway results in improved regulation of transport in chronic colitis. In addition, since the implications of such a strategy for the inflammatory reaction itself are little

explored in experimental colitis [595], there are two valuable outcomes of this experiment. A double approach was followed, i.e. blockade of the DP_2 receptor with ramatroban (also involving thromboxane receptor), and inhibition of the H-PGDS synthase HQL-79 (we additionally contacted Pfizer to obtain a totally specific DP_2 blocker, known as 'compound A', and we are currently awaiting the evaluation of our request). We tested Ramatroban (10 mg/kg) and HQL-79 (1mg/kg) as a pretreatment protocol in rat TNBS colitis. The results obtained show a significant amelioration of colonic myeloperoxidase activity and a number of other inflammatory markers, including S100A8, IL-1β, INOS, MCP-1 or TNF. Despite the positive, albeit limited, effect on inflammation, there was no effect on transport. In contrast, ramatroban had only a minimal effect on colitis. Because HQL-79 beneficial effect is predicted to occur via reduced DP_2 activation, the lack of therapeutic benefit with ramatroban is inconsistent. It is possible that the dose assayed is insufficient. At any rate, the dose used by us had a significant but totally unexpected impact on transport, since it was surprisingly associated with markedly reduced basal and carbachol induced secretion. Although we have no clear explanation for these transport data, one possibility is that chronic DP_2 receptor blockade by ramatroban augments receptor sensitivity, which would in turn cause intense unopposed activation by PGD_2 in the Ussing chambers, after residual ramatroban has diffused away. This possibility is being currently studied. This presumably would not happen with HQL-79 because of a lower overall effect (one of the synthases is not blocked) and because of the need to synthetize PGD_2 in vitro to produce the same effect.

In summary, our results indicate that the disturbances in ion transport, and specifically ion secretion, observed in rat TNBS colitis are chiefly of regulatory nature and associated with low intracellular levels of cAMP in the epithelium. In turn, this deficit in cAMP production has been related to cytokine induced changes in the expression of adenylate cyclase isoforms and to the modulatory influence of PGD_2 on epithelial cells acting on DP_2 receptors. Our data do not support a major role of opioids, nitric oxide or changes in the transportome. However, because each of these factors has been studied separately, it is quite possible that additive minor effects may converge into the intense inhibition of ion secretion observed in vivo. While the response of human colon to PGD_2 is clearly different from that of the rat colon in normal conditions, our data suggest that this eicosanoid may play a significant role in IBD, although this requires further experimentation. We do not want to end the Discussion without mentioning that a number of results have been left out of this doctoral thesis because of an incomplete development, but we expect that they will see the light of day in the near future and help clarify our view of the epithelial disturbances that take place in IBD.

CONCLUSIONS

1. In rat TNBS colitis the epithelial expression of adenylate cyclase 5, 6 and 9 is downregulated. This effect may be caused by proinflammatory cytokines (IL-1β, TNFα and IFNγ) and contribute to the defect in ion secretion. The novel system of organoid culture constitutes a valuable non tumoral model to validate changes in adenylate cyclase expression.

2. Increased production of PGD_2 in rat TNBS colitis activates epithelial DP2 receptors, with subsequent increment Gi protein function, decreased intracellular cAMP levels and reduced basal ion secretion. Neither NO nor endogenous opioids seem to play a substantial role at this level.

3. Changes in the enterocyte transportome do not seem to underlie the defects in ion transport in rat TNBS colitis.

4. The effect of PGD_2 in the human uninflamed colon is substantially different to that in the rat colon, due to differences in the expression of transducing receptors. However, PGD_2 might inhibit ion secretion in human IBD samples in a similar way to that observed in rat TNBS colitis.

5. The PGD_2/DP_2 axis plays a pathogenic role in rat TNBS colitis. Inhibition of PGD_2 synthesis or DP_2 receptor occupation does not reverse the defect in ion secretion.

CONCLUSIONES

1. En el modelo de colitis experimental inducida por TNBS en rata, la expresión epitelial de adenilato ciclasa 5,6 y 9 está inhibida. Este efecto puede deberse a citoquinas proinflamatorias (IL-1β, TNFα and IFNγ) y contribuye al defecto en la secreción iónica. El novedoso sistema de cultivo de organoides constituye un valioso modelo de validación no tumoral para dichos resultados.

2. El aumento de la producción de PGD_2 en la colitis por TNBS en rata activa receptores DP_2, con el subsecuente incremento en la función de la proteína Gi, reducción intracelular de los niveles de cAMP e inhibición de la secreción iónica basal. Ni el óxido nítrico no los opiáceos endógenos parecen jugar un papel sustancial a este nivel.

3. Cambios en el transportoma del enterocito no explican el estado de inhibición iónica en la colitis de rata por TNBS.

4. El efecto de PGD_2 en colon no inflamado de humano es sustancialmente diferente al colon de rata, debido a diferencias de expresión en la transducción receptorial. Sin embargo, PGD_2 puede inhibir la secreción iónica en biopsias de pacientes con IBD de manera similar que lo observado en colitis de rata por TNBS.

5. El eje PG_2/DP_2 juega un rol patogénico en la colitis de rata por TNBS. La inhibición de la síntesis de PGD_2 o la ocupación del receptor DP_2 no reviernte el defecto en secreción iónica.

ABBREVIATIONS

For a better understanding the main abbreviations used in this thesis are presented below:

5-ASA	5-aminosalicylic acid
8-Br-cAMP	8-bromoadenosine 3',5'-cyclic monophosphate
ABC	ATP binding cassette family
AE2	Basolateral anion exchanger 2 or Slc4a2
AEC	Aminoethyl carbazole substrate
AC	Adenylate cyclase
Aco2	Aconitase 2
Aldob	Aldolase B gene
AQPs	Aquaporins
ATG16L1	Autophagy related 16-like 1 gene
ATP	Adenosine triphosphate
BBM	Brush border membrane
CA	Carbonic anhydrase
CaM	Calmodulin kinase
cAMP	Cyclic adenosine monophosphate, 3'5'-cyclic AMP
CD	Crohn's disease
cGMP	Cyclic guanosine monophosphate, 3'5'-cyclic GMP
CF	Cystis fibrosis
CFTR	Cystic Fibrosis Transmembrane conductance Regulator or ABCC7
CLD	Chloride-losing diarrhea
Cs	Citrate synthase
Cxcl1	Chemokine (C-X-C motif) ligand 1
DAG	Diacylglycerol
Dlat	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex
Dld	Dihydrolipoyl dehydrogenase
DP	Prostaglandin D receptor
DP_1	PGD_2 receptor type 1 or D-prostanoid receptor
DP_2	PGD_2 receptor type 2 or CRTH2
DRA	Down Regulated in Adenoma or Slc26a3
DSS	Dextran sodium sulfate
DTT	Dithiothreitol
EGF	Epidermal growth factor
ENaC	Epithelial sodium channel
eNOS	Endothelial nitric oxide synthase
EP	Prostaglandin E receptor
FBS	Fetal bovine serum
FITC-D	Fluorescein isothiocyanate-dextran
FLAP	5-lipoxygenase activating protein
FP	Prostaglandin F receptor
GADHP	Glyceraldehyde-3-phosphate dehydrogenase
GALT	Gut-associated lymphoid tissue
GC	Guanylate cyclase
GC-C	Guanylate cyclase C
GCS	Glucocorticosteroids
GPCR	G-protein-coupled receptor
Gro	Growth-regulated protein or Cxcl2
GTP	Guanosine triphosphate
GWAS	Genome-wide association studies

H-PGDS	Hematopoietic PGD Synthase or spleen-type PGDS
H&E	Hematoxylin and eosin stain
HBSS	Hank´s balanced salt solution
IAP	Intestinal alkaline phosphatase
IBD	Inflammatory bowel disease
IBMX	3-isobutylmethylxanthine
IEL	Intraepithelial lymphocytes
IFNγ	Interferon γ
IL-1β	Interleukin 1β
IHC	Immunohistochemistry
iNOS	Inducible Nitric Oxide Synthase
IL-10	Interleukin 10
IP	Prostaglandin I receptor
IP_3	Inositol 1,4,5-trisphosphate
IRGM	Immunity-related GTPase family M gene
Isc	Short-circuit current
JAK2	Janus kinase 2
KLF4	Krüppel-like factor 4
lincRNA	Large intervening non-coding RNA
L-NIL	L-N6-(1-iminoethyl) lysine
Lbp	Lipopolysaccharide binding protein
LDH	Lactate dehydrogenase
LOX	Lipoxygenase
L-PGDS	Lipocalin-Type PGD Synthase
LT	Leukotrienes
Mcp-1 Ccl2	Monocyte chemotactic protein-1 or
miRNA	MicroRNA
MPO	Myeloperoxidase
MSDs	Membrane-spanning domains
Na^+/K^+-ATPase	Na+ pump
NBDs	Nucleotide-binding domains
Ndufs1 protein 1	NADHdehydrogenase (ubiquinone) Fe-S
nNOS	Neural nitric oxide synthase
NF-κB	Nuclear Factor-kappa B
Nfkb1	Nuclear factor of kappa light chain gene
NHE1	Na^+/H^+ exchanger 1 or Slc9a1
NHE2	Na^+/H^+ exchanger 2 or Slc9a2
NHE3	Na^+/H^+ exchanger 3 or Slc9a3
NHERF	Sodium/proton exchanger regulatory factor
NHERF1	Sodium/proton exchanger regulatory factor 1
NHERF2	Sodium/proton exchanger regulatory factor 2
NKCC1	$Na^+/K^+/2Cl^-$cotransporter or Slc12a2
NO_2^-	Nitrite
NO_3^-	Nitrate
NKCC1	$Na^+/K^+/2Cl^-$ cotransporter
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NO	Nitric oxide
NOS	Nitric Oxide Synthases
NSAID	Nonsteroidal antiinflammatory drugs
PAT1	Slc26a6 or CFEX
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
Pdha1 subunit alpha	Pyruvate dehydrogenase E1 component

Pdhb	Pyruvate dehydrogenase E1 component
subunit beta	
PDZ	PSD95/Dlg/ZO1
PDZK1	NHERF3
PDZK2	NHERF4
PTX	Pertussis Toxin
PGs	Prostaglandins
PGDS	Prostaglandin D_2 synthases
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGI_2	Prostacyclin
PIP_2	Phospholipid phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PUFAs	N-3 polyunsaturated fatty acids
RIPA	Radioimmunoprecipitation assay buffer
S100A8	S100 calcium binding protein A8 or
Calgranulin A	
SAT3	Streptothricin-acetyltransferase gene
SGLTs	Na^+-glucose-linked transporters or Slc5
SCFA	Short chain fatty acids
Slc	Solute carrier
Slc6, Slc38	Na^+/amino acid contransporters
SNP	Single nucleotide polymorphism
SRM	Selected reaction monitoring
Sucla2	Succinate-CoA ligase
TJ	Tight junctions
Tlr2	Toll-like receptor 2
TNBS	Trinitrobenzenesulfonic acid
TNFα	Tumor necrosis factor α
TP	Thromboxane A receptor
TYK2	Tyrosine kinase 2 gene
TX	Thromboxane
UC	Ulcerative colitis

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