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The role of epithelial $P2Y_2$ and $P2Y_4$ receptors in the regulation of intestinal chloride secretion

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1 UTP-induced chloride secretion by the intestinal mucosa mounted in Ussing chambers was assessed by measurement of the short-circuit current (I_{sc}) in the presence of phloridzin in the case of jejunum or amiloride in the case of colon to eliminate any contribution of electrogenic Na^+ movement to the net ionic transport. Since we have previously demonstrated the absence of chloride-secretory response to apical UTP in the jejunum from $P2Y_4$-null mice, in the present study we studied the response to basolateral UTP in the jejunum and to either apical or basolateral UTP in the colon, in both $P2Y_2$- and $P2Y_4$-deficient mice.

2 In the jejunum, the chloride-secretory response to basolateral UTP was partially reduced in both $P2Y_2$- (40%) and $P2Y_4$- (60%) null mice.

3 In the colon, both apical or basolateral UTP increased the I_{sc}. That response was abolished in a chloride-free medium.

4 The colonic chloride-secretory response to either basolateral or apical UTP was abolished in $P2Y_4$-deficient mice, but not significantly affected in $P2Y_2$-deficient mice. The chloride-secretory response to forskolin was potentiated by prior basolateral addition of UTP and this potentiation was abolished in $P2Y_4$-null mice.

5 The jejunum of mice homozygous for the ΔF508 mutation of cystic fibrosis transmembrane conductance regulator was responsive to UTP, but the magnitude of that response was smaller than in the wild-type littermates.

6 In conclusion, the $P2Y_4$ receptor fully mediates the chloride-secretory response to UTP in both small and large intestines, except at the basolateral side of the jejunum, where both $P2Y_2$ and $P2Y_4$ receptors are involved.
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Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; I_{sc}, short-circuit current (expressed as $\mu A\,cm^{-2}$); PCR, polymerase chain reaction

Introduction

It is known that ATP and UTP modulate epithelial electrolyte transport in small and large intestines. Indeed, effects on Cl^- secretion, K^+ secretion and Na^+ reabsorption have been demonstrated either in mouse, rat or guinea-pig tissues or in cultured human cells (Caco-2, T84), mostly using Ussing chambers (Inoue *et al*., 1997; Leipziger *et al*., 1997; Kerstan *et al*., 1998; Cressman *et al*., 1999; McAlroy *et al*., 2000; Lazarowski *et al*., 2001; Smitham & Barrett, 2001; Kunzelmann & Mall, 2002; Yamamoto & Suzuki, 2002; Köttgen *et al*., 2003; Leipziger, 2003). As bacterial invasion of epithelia induces the release of nucleotides, this response may play a role in host defense and infectious diarrhea (McNamara *et al*., 2001; Crane *et al*., 2002; Van Nhieu *et al*., 2003). Although the response to ATP is partially mediated by its degradation into adenosine and the activation of adenosine receptors (Bucheimer & Linden, 2004), the action of UTP can be explained only by the activation of P2Y receptors. Pharmacological responses to UTP can be mediated by either $P2Y_2$ or $P2Y_4$ receptors, and no selective antagonist is available to discriminate between them (Wildman *et al*., 2003). In the airway epithelium (nasal epithelial cells, trachea), the responses to UTP ($[Ca^{2+}]_i$ increase, chloride secretion) were abolished in $P2Y_2^{-/-}$ mice (Cressman *et al*., 1999; Homolya *et al*., 1999). However, the chloride-secretory response to apical UTP was maintained in the jejunum of $P2Y_2$-null mice. We recently generated $P2Y_4$-null mice in which that response was abolished (Robaye *et al*., 2003). Extracellular nucleotide signaling in epithelia is a complex process and distinct receptors can be involved in different tissues, but also in the apical *versus* basolateral membrane (Dubyak, 2003). In the present study, we have

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systematically investigated the respective roles of $P2Y_2$ and $P2Y_4$ receptors in the apical *versus* basolateral response to UTP in both jejunum and colon.

Methods

Mice

$P2Y_4$-null mice have been generated in the mixed CD1-129SV genetic background, as described (Robaye *et al*., 2003). $P2Y_2^{-/-}$ mice initially in the B6D2 background (Homolya *et al*., 1999), generously given by Dr BH Koller, were outbred in the 129SV background (Matos *et al*., 2005). Mice homozygous for the ΔF508 mutation of cystic fibrosis transmembrane conductance regulator (CFTR) in the 129/FVB background (van Doornink *et al*., 1995) were a generous gift of Dr BJ Scholte. Genotyping was performed by PCR on DNA extracted from clipped tails and using the following primers:

$P2Y_2$: triple primer PCR

forward primer 5′–3′	*reverse primer 5′–3′*	*Allele*
GTCACGCGCACCC TCTACTA	TCGGGTGCACTGCC TTTCTT	Wild type
GGGGAACTTCCTG ACTAGG	TCGGGTGCACTGC CTTTCTT	Mutated

$P2Y_4$: two independent PCR

forward primer 5′–3′	*reverse primer 5′–3′*	*Allele*
AGTAGAGGTTCCA GTAGAAA	GACTCCTTGCTAT TCACA	Wild type
CGAAGTTATATTAA GGGTTC	TAATCGGTCACC CTCA	Mutated

The genotyping of ΔF508 mice (CF mice) was performed at 21 days of age using Taqman quantitative PCR multiplex analysis of tail clip DNA. For each type of experiment, littermates were used as controls. In the case of $P2Y_4$, an X-linked gene, only males were used. $P2Y_2^{-/-}$ and $P2Y_4^{0/-}$ mice and their littermates were maintained on a standard diet in conventional facilities. ΔF508 mice and their littermates were weaned to a liquid diet (Peptamen®, Nestlé Clinical Nutrition, France) and the colony was maintained in a pathogen-free status. Mice were kept in a 12-h light–dark cycle. The studies were approved by the local Ethics Committee of Animal Welfare and conformed to the Guide for the Care and Use of Laboratory Animals adopted and promulgated by the US National Institutes of Health.

Ussing chambers measurements

Briefly, mice aged 2–5 months were killed by intraperitoneal pentobarbital ($10\,mg\,kg^{-1}$). The midportion of the jejunum, extending 10 cm after the ligament of Treitz, or the distal 2 cm portion of the colon were dissected, opened and washed with Krebs bicarbonate solution. The mucosa was stripped from the adjacent muscularis layer and sealed on the basolateral side to a fixation ring with an opening diameter of 3 mm. This ring was placed between the halves of an Ussing chamber. KCl electrodes, connected to the solution via a short agar bridge, were used for measuring the potential difference and passing current. Impedance analysis was used to determine the resistance of the epithelium and bathing solution between the voltage electrodes as described recently (Ghanem *et al*., 2005). In this analysis, the epithelium can be represented by a lumped model consisting of a parallel circuit of a capacitance and resistance in series with the solution resistance between the voltage electrodes (R_{sol}). The resistance shunting the capacitance represents the transepithelial resistance (R_{epi}). The impedance analysis provides an evaluation of R_{sol} and $R_{sol}+R_{epi}$. In this series of experiments, the mean values were: $R_{sol}=25\pm3\,Ohm\,cm^2$ ($n=29$), $R_{epi}=14\pm2\,Ohm\,cm^2$ ($n=12$) for jejunal epithelium and $R_{epi}=46\pm6\,Ohm\,cm^2$ ($n=17$) for colonic epithelium. Since R_{sol} attenuates the current recorded by the voltage clamp ($I_{sc\,m}$), the actual short-circuit current ($I_{sc\,id}$) expected for an ideal voltage clamp across the epithelium was calculated as: $I_{sc\,id}=I_{sc\,m}\,(R_{epi}+R_{sol})/R_{epi}$. These $I_{sc\,id}$ values are equivalent to the net ionic flow through the epithelium and are subsequently abbreviated as I_{sc}.

The volume of each compartment bathing the jejunal or colonic mucosa was 2 ml and Krebs bicarbonate solution pre-equilibrated with a gas mixture of 5% CO_2–95% O_2 at 37°C was flowing in each compartment at a rate of $20\,ml\,min^{-1}$. The composition of the Krebs bicarbonate solution was the following in mM: Na: 140; K: 5.2; Mg: 1.2, Ca: 1.2; Cl: 120; PO_4: 2.8; HCO_3: 25; glucose: 11.5; pH 7.4. In some experiments, chloride was isoosmotically replaced by gluconate.

Study of Cl^--secretory response in jejunum and colon

The I_{sc} can be divided into two components: (1) a sodium absorptive component linked to the apical sodium–glucose cotransporter of villi enterocytes in the jejunum or to the apical epithelial sodium channel (ENaC) in surface colonocytes of the large intestine and (2) a chloride-secretory component linked to the existence of apical chloride channels in crypt cells of both jejunum and colon. The first component can be eliminated by addition of 1 mM phloridzin or 100 μM amiloride to the apical bath in the jejunum or colon, respectively. The small magnitude of the amiloride effect in our colon experiments is likely to be explained by the limited number of ENaC in non-sodium-restricted mice. The second component of the I_{sc} was quantitatively accounted by chloride secretion as it was abolished in chloride-free solutions in both jejunum and colon. The maneuvres of stripping the mucosa from its adjacent muscularis and its mounting in a small Ussing chamber induce the release of prostaglandins, a potential stimulus to chloride secretion that could mask other stimuli, and therefore chloride secretion was assessed as the I_{sc} following addition of phloridzin or amiloride to the apical side as well as of indomethacin (100 μM) to both bathing media. UTP was added, at 100 μM, to the apical or basolateral solution, unless otherwise stated. Forskolin was always added to the basolateral solution at the concentration of 10 μM in ethanol 0.1%; this concentration of ethanol does not affect I_{sc} (data not shown). The increase in I_{sc} (expressed in $\mu A\,cm^{-2}$) was calculated as the difference between the basal current and the peak current obtained within 15 min of addition of UTP or forskolin. Data are expressed as mean ± s.e.m. Statistical

analysis was performed using the unpaired *t*-test and a *P*-value <0.05 was considered significant.

Materials

UTP, indomethacin, forskolin, phloridzin and amiloride were purchased from Sigma (Merelbeke, Belgium).

Results

Chloride secretion by jejunal mucosa

In order to examine the effect of UTP on intestinal chloride secretion, pieces of jejunal mucosa, stripped of muscle layers, were placed in Ussing chambers and pre-exposed to phloridzin (1 mM) and indomethacin (100 μM). Although in these conditions no true steady state was reached within the time of study, a slow 'predictable' drift was usually obtained so that acute change in I_{sc} elicited by a given agent could be unquestionably ascribed to this stimulatory agent. Addition of UTP (100 μM) to the basolateral side of mice jejuna increased I_{sc} (Figure 1). The magnitude of that response was significantly different in control mice from different genetic background: $69\pm6\,\mu A\,cm^{-2}$ in CD1 mice and $27\pm3\,\mu A\,cm^{-2}$ in 129SV mice (mean $\pm$ s.e.m. of five animals). Within the same strain, the $P2Y_4^{0/+}$ mice, the response to basolateral UTP was greater than the response to apical UTP, showing a mean value of $69\pm6\,\mu A\,cm^{-2}$ as compared to the $17\pm5\,\mu A\,cm^{-2}$ value previously reported for the apical response (Robaye *et al*., 2003). The effect of basolateral UTP was decreased, but not completely abolished, in $P2Y_4^{0/-}$ mice (Figure 1): $30\pm7\,\mu A\,cm^{-2}$ (mean $\pm$ s.e.m. of five animals), or a 57% inhibition. That response was also decreased in $P2Y_2^{-/-}$ mice as compared to their control littermates: $17\pm2\,\mu A\,cm^{-2}$ (mean $\pm$ s.e.m. of five animals), or a 37% decrease. We confirmed that the response to apical UTP was maintained in $P2Y_2^{-/-}$ mice, as reported previously (Cressman *et al*., 1999) (data not shown).

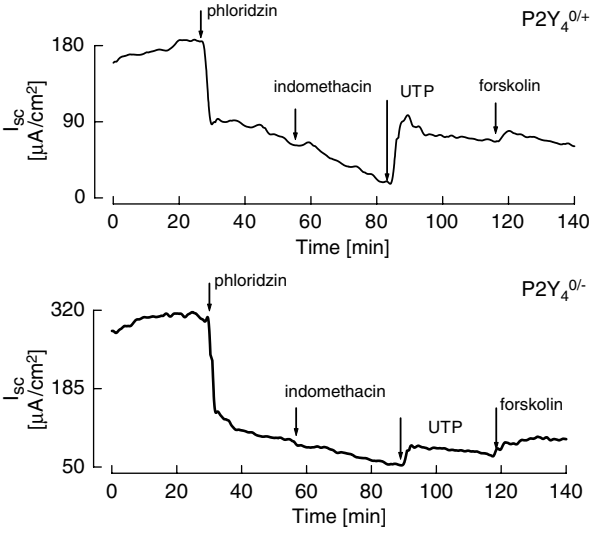

Figure 1 Stimulation of I_{sc} by basolateral UTP in the jejunum of control (upper panel) and $P2Y_4$-null (lower panel) mice. Sodium–glucose cotransport activity was inhibited by adding 1 mM phloridzin to the apical solution. Indomethacin (100 μM) was added to both sides. UTP (100 μM) and forskolin (10 μM) were added to the basolateral bath.

Chloride secretion by colonic mucosa

In the distal colon of $P2Y_4^{0/+}$ mice, apical and basolateral UTP increased the I_{sc} with a magnitude about two-fold smaller than that in the jejunum (Figure 2, Table 1). The responses to both apical and basolateral UTP were abolished in the colon of $P2Y_4^{0/-}$ mice (Figure 2, Table 1). In $P2Y_2^{-/-}$ mice, these UTP-mediated responses were not significantly different from those of their control littermates (data not shown). The response to basolateral forskolin after apical addition of UTP was not different in $P2Y_4^{0/-}$ and control mice (Table 1). On the contrary, pre-exposure to basolateral UTP amplified the

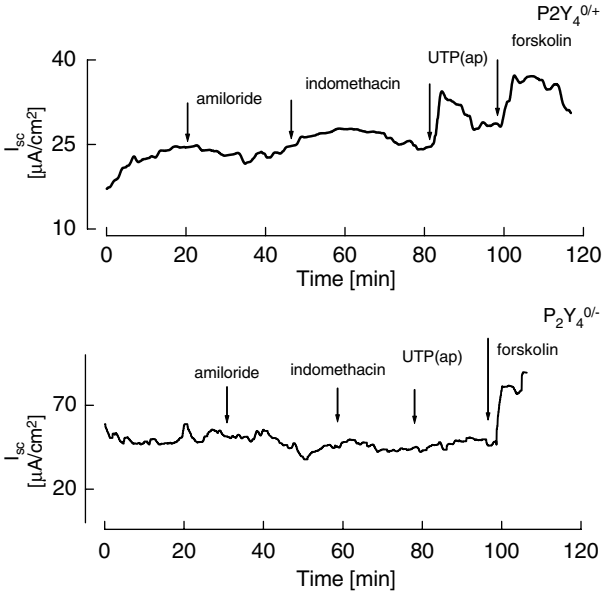

Figure 2 Stimulation of I_{sc} by apical UTP in the colon of control (upper panel) and $P2Y_4$-null (lower panel) mice. Sodium absorption was inhibited by adding amiloride (100 μM) to the apical solution. The small magnitude of the amiloride effect is probably related to the limited number of ENaC in non-sodium-restricted mice. Indomethacin (100 μM) was added to both sides. UTP (100 μM) was added to the apical bath and forskolin (10 μM) to the basolateral one.

Table 1 Increase of the I_{sc} in mouse colon stimulated by UTP: comparison between control and $P2Y_4$-deficient mice

	$P2Y_4^{0/+}$	$P2Y_4^{0/-}$	*N(P)*
UTP (apical)	11 $\pm$ 1	1 $\pm$ 1	5 (<0.001)
Forskolin (basolateral)	26 $\pm$ 7	24 $\pm$ 5	5 (NS)
UTP (basolateral)	23 $\pm$ 3	2 $\pm$ 1	5 (<0.001)
Forskolin (basolateral)	50 $\pm$ 10	25 $\pm$ 3	5 (<0.05)
Forskolin (no UTP)	21 $\pm$ 3	22 $\pm$ 3	5 (NS)

The increase in I_{sc} (expressed in $\mu A\,cm^{-2}$) is calculated as the difference between basal current and peak current obtained within 15 min of addition of UTP (100 μM) or forskolin (10 μM).

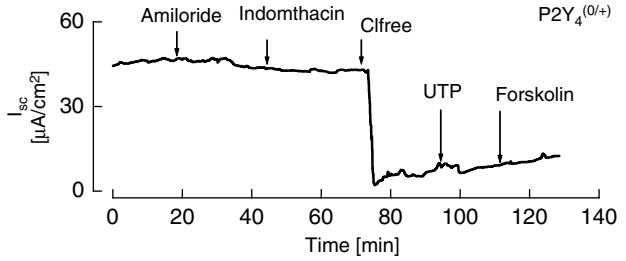

Figure 3 Effect of replacement by a chloride-free medium on the stimulation of I_{sc} by basolateral UTP and forskolin in the colon. In the chloride-free medium, chloride was replaced by isoosmotic gluconate. Amiloride and indomethacin were added as in Figure 2.

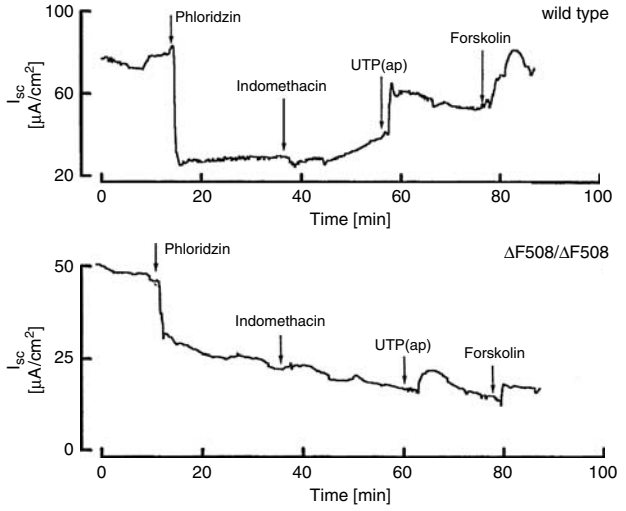

Figure 4 Stimulation of Cl^- secretion by apical UTP in the jejunum of control mice and mice homozygous for the ΔF508 mutation. Sodium–glucose cotransport activity was inhibited by adding 1 mM phloridzin to the apical solution. Indomethacin (100 μM) was added to both sides. UTP (100 μM) was added to the apical bath and forskolin (10 μM) to the basolateral one.

response to forskolin and that potentiation was abolished in $P2Y_4^{0/-}$ mice (Table 1). In a chloride-free medium, the resting value of the I_{sc} was greatly reduced and the response to apical UTP as well as basolateral forskolin was abolished (Figure 3).

Jejunal chloride secretion in ΔF508 mice

In the jejunum of mice homozygous for the ΔF508 mutation of CFTR, an increase in I_{sc} in response to apical UTP was present, though diminished as compared to wild-type littermates (Figure 4). It was $15 \pm 2\,\mu A\,cm^{-2}$ as compared to $31 \pm 6\,\mu A\,cm^{-2}$ (mean ± s.e.m. of five animals). The response to forskolin was also decreased in ΔF508/ΔF508 as compared to wild-type littermates: $19\,\mu A\,cm^{-2}$ *versus* $45\,\mu A\,cm^{-2}$ (mean ± s.e.m. of five animals).

Discussion

We have recently developed a new method to compute the actual values of the I_{sc} across leaky epithelia using impedance analysis to estimate the transepithelial resistance as well as the resistance of the bathing solution (Ghanem *et al*., 2005). Although such method may appear complicated, it should be emphasized that it is the only way to correct the recorded I_{sc}. The currents are indeed underestimated by the resistance of the bathing solution in series with the epithelium. For leaky epithelia, such as in jejunum and colon, this correction is important because the transepithelial resistance is in the same range as the solution resistance. Using this method, we demonstrate here the occurrence of chloride secretion across native murine colonic epithelium, a phenomenon so far observed only in cultured colonic epithelia (Kunzelmann & Mall, 2002). Furthermore, thanks to this method, we delineate the respective roles of $P2Y_4$ and $P2Y_2$ receptors in the chloride-secretory response to apical and basolateral UTP in jejunum and colon.

We have previously demonstrated that the chloride-secretory response to apical UTP is mediated entirely by the $P2Y_4$ receptor in the murine jejunum (Robaye *et al*., 2003). We now show that the situation is different at the basolateral side, where both $P2Y_4$ and $P2Y_2$ receptors appear to play a role. There are precedents for such asymmetry in the literature (Dubyak, 2003). Differences between the responses to apical and basal UTP have been noticed previously in human colonic cells (Smitham & Barrett, 2001). It was recently shown that $P2Y_2$, $P2Y_6$ and $P2Y_{11}$ receptors are present on the luminal membrane of human nasal epithelial cells, whereas only $P2Y_2$ receptors are found on the basolateral membrane (Kim *et al*., 2004). This asymmetry is also consistent with the involvement of different effector mechanisms activated by nucleotides: for instance, CFTR on the luminal side and Na^+–K^+–$2Cl^-$ cotransporter (NKCC) on the basolateral side (Köttgen *et al*., 2003; Shin *et al*., 2004). It was recently shown that, following transfection in Madin–Darby canine kidney cells, the $P2Y_1$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{14}$ receptors reside at the basolateral membrane, whereas $P2Y_2$, $P2Y_4$ and $P2Y_6$ are expressed at the apical membrane (Wolff *et al*., 2005). The authors suggested that the polarized targeting of P2Y receptor subtypes is not a function of the type of epithelial cells, and thus extrapolated that $P2Y_2$ and $P2Y_4$ are always apical. In contradiction to this oversimplistic rule, expression of both apical and basolateral $P2Y_2$ has been characterized in diverse epithelia (Homolya *et al*., 1999). Our results also do not support this extrapolation, since we obtained evidence that in jejunum and colon functional $P2Y_4$ receptors are expressed in both basolateral and apical membranes, whereas in jejunum functional $P2Y_2$ receptors are only present on the basolateral side.

We have observed that UTP increases I_{sc} also in the colon: abolition of that response in chloride-free medium indicates that UTP stimulates chloride-secretion. A chloride-secretory response to ATP/UTP has been reported previously in human colonic cell lines, Caco-2 (Inoue *et al*., 1997) and T84 (Smitham & Barrett, 2001), but not in the native human or murine colonic mucosa (Kunzelmann & Mall, 2002; Leipziger, 2003). The response to both apical and basolateral UTP (100 μM) was abolished in $P2Y_4^{0/-}$ and maintained in $P2Y_2^{-/-}$ mice. The potency of UTP is similar at recombinant murine $P2Y_2$ and $P2Y_4$ receptors, with EC_{50} below 1 μM, and 100 μM UTP produces a maximal effect on I_{sc} in the trachea (where $P2Y_2$ is expressed) and an almost maximal effect in the jejunum (where $P2Y_4$ is expressed) (Cressman *et al*., 1999;

Lazarowski *et al*., 2001). Therefore, although concentrations of UTP $>100\,\mu$M were not tested, it can be safely concluded that the colonic responses to UTP involve exclusively the P2Y$_4$ subtype and not the P2Y$_2$ one.

Exposure to basolateral UTP prior to basolateral forskolin amplified the forskolin response in the colon, and this was totally dependent on the P2Y$_4$ receptor. It is well known that cAMP and $[Ca^{2+}]_i$ can regulate Cl^- secretion in a synergistic way: in colonic epithelial cells, cAMP directly activates apical CFTR, while Ca^{2+} stimulates basolateral SK4 K^+ channels and thereby increases the driving force for Cl^- secretion (Kunzelmann & Mall, 2002). Such phenomenon did not occur when UTP was added at the luminal side. This is consistent with a partially compartmentalized signaling. Compartmentalized calcium signaling has been described in human nasal epithelial cells (Paradiso *et al*., 1995; Shin *et al*., 2004) and equine sweat gland cells (Wong & Ko, 2002). In Calu-3 cells, activation of apical A_{2B} receptors by adenosine induced cAMP signaling that remained restricted to the apical membrane (Huang *et al*., 2001).

It was reported previously that the jejunal response to apical UTP was abolished in CFTR$^{-/-}$ mice, in agreement with the concept that CFTR is the only chloride transporter present in the intestine apical membrane (Lazarowski *et al*., 2001). In mice homozygous for the ΔF508 mutation of CFTR, a response to apical UTP was clearly detectable, though diminished, as compared to wild-type littermates. The response of the ΔF508/ΔF508 mice to forskolin was previously reported to be about 30% of that observed in control mice (Van Doornink *et al*., 1995). These results are consistent with the observation of a low expression of ΔF508-CFTR in the murine intestine, where it can be stimulated by pharmacological agents (Van Doornink *et al*., 1995; French *et al*., 1996; Steagall & Drumm, 1999). It would therefore be tempting to speculate that the P2Y$_4$ receptor could be a pharmacological target to treat the intestinal abnormalities in the large number of patients harbouring the ΔF508 mutation. However, in other ΔF508/ΔF508 mice, chloride secretion in the jejunum was totally unresponsive to cAMP (Zeiher *et al*., 1995). Moreover, there are discrepancies on the membrane expression of human ΔF508-CFTR in the literature (Kälin *et al*., 1999; Bronsveld *et al*., 2001). A very recent study, based on both immunochemistry and Ussing chambers measurements, failed to detect any expression of the protein and any functional response to carbachol in rectal biopsies (Mall *et al*., 2004). Although it might be argued that rectum is not representative of jejunum and that the UTP signaling mechanisms may be partially different from those of carbachol, this report suggested that this therapeutic possibility is not realistic. On the other hand, the role of the P2Y$_4$ receptor in infectious diarrhea remains to be evaluated, especially in view of the recent reports showing that epithelial invasion by enteropathogenic bacteria induces the release of nucleotides (McNamara *et al*., 2001; Crane *et al*., 2002; Van Nhieu *et al*., 2003).

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