K^+ secretion activated by luminal $P2Y_2$ and $P2Y_4$ receptors in mouse colon

J. E. Matos[1], B. Robaye[2], J. M. Boeynaems[2,3], R. Beauwens[4] and J. Leipziger[1]

[1] *Institute of Physiology and Biophysics, The Water and Salt Research Center, University of Aarhus, Denmark*
[2] *Institute of Interdisciplinary Research, Institute of Biology and Molecular Medicine, Université Libre de Bruxelles, Gosselies, Belgium*
[3] *Laboratory of Medical Chemistry, Erasme Hospital, Université Libre de Bruxelles, Belgium*
[4] *Laboratory of Physiopathology, School of Medicine, Université Libre de Bruxelles, Belgium*

Extracellular nucleotides are important regulators of epithelial ion transport, frequently exerting their action from the luminal side. Luminal P2Y receptors have previously been identified in rat distal colonic mucosa. Their activation by UTP and ATP stimulates K^+ secretion. The aim of this study was to clarify which of the P2Y receptor subtypes are responsible for the stimulated K^+ secretion. To this end $P2Y_2$ and $P2Y_4$ knock-out mice were used to measure distal colonic ion transport in an Ussing chamber. In mouse (NMRI) distal colonic mucosa, luminal UTP and ATP with similar potency induced a rapid and transient increase of the transepithelial voltage (V_{te}) (UTP: from -0.81 ± 0.23 to 3.11 ± 0.61 mV, $n = 24$), an increase of equivalent short circuit current (I_{sc}) by 166.9 ± 22.8 μA cm^{-2} and a decrease of transepithelial resistance (R_{te}) from 29.4 ± 2.4 to 23.5 ± 2.0 Ω cm^2. This effect was completely inhibited by luminal Ba^{2+} (5 mM, $n = 5$) and iberiotoxin (240 nM, $n = 6$), indicating UTP/ATP-stimulated K^+ secretion. RT-PCR analysis of isolated colonic crypts revealed $P2Y_2$, $P2Y_4$ and $P2Y_6$ specific transcripts. The luminal UTP-stimulated K^+ secretion was still present in $P2Y_2$ receptor knock-out mice, but significantly reduced (ΔV_{te}: 0.83 ± 0.26 mV) compared to wild-type littermates (ΔV_{te}: 2.08 ± 0.52 mV, $n = 9$). In $P2Y_4$ receptor knock-out mice the UTP-induced K^+ secretion was similarly reduced. Luminal UTP-stimulated K^+ secretion was completely absent in $P2Y_2$/$P2Y_4$ double receptor KO mice. Basolateral UTP showed no effect. In summary, these results indicate that both the $P2Y_2$ and $P2Y_4$ receptors are present in the luminal membrane of mouse distal colonic mucosa, and stimulation of these receptors leads to K^+ secretion.

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Corresponding author J. Leipziger: Ole Worms Allé 160, 8000 Aarhus C, Denmark. Email: leip@fi.au.dk

P2 or nucleotide receptors are strongly expressed in all transporting epithelia and regulate ion secretion and absorption (Dubyak, 1999; Lazarowski & Boucher, 2001; Leipziger, 2003). Frequently, these membrane receptors are expressed both in the basolateral and luminal membranes. The luminal expression of P2 receptors is a phenomenon unique to epithelial organs (Leipziger, 2003). The activation of P2 receptors by extracellular nucleotides like ATP or UTP commonly influences ion transport processes. As a general theme, P2 receptor activation can trigger two different effects: (1) activation of ion (and water) secretion, and (2) inhibition of Na^+ absorption. Pro-secretory effects include the activation of Cl^- secretion in human respiratory epithelium (Mason *et al.* 1991), activation of HCO_3^- secretion in mouse gallbladder epithelium (Clarke *et al.* 2000) and stimulation of K^+ secretion in rat distal colon (Kerstan *et al.* 1998), *Necturus* gallbladder (Cotton & Reuss, 1991) and human airway epithelium (Clarke *et al.* 1997). The inhibition of Na^+ absorption was found in different steroid-sensitive epithelia and involves ENaC channels (Mall *et al.* 2000; Lehrmann *et al.* 2002*a*,*b*).

Mammalian P2 receptors are subdivided into metabotropic P2Y and ionotropic P2X receptors. Currently, eight different P2Y have been identified: five of them are linked to G_q proteins ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$) and three have been shown to be G_i-coupled ($P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) (Abbracchio *et al.* 2003). There is clear evidence for expression of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in intact epithelial tissues (Ralevic & Burnstock, 1998; Dubyak, 2003; Leipziger, 2003). In addition, seven different P2X receptors are described in mammalian cells ($P2X_{1-7}$) (North, 2002).

In many epithelial tissues luminal UTP is a lead agonist in the alteration of ion transport (Leipziger, 2003), and exclusively stimulates the $P2Y_2$ and the $P2Y_4$ receptors

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Table 1. Summary of different mice used and their respective genetic background

Mice used	Genetic background
NMRI	NMRI
Original $P2Y_2$ KO	B6D2
$P2Y_2$ WT and KO	B6D2/SV129
$P2Y_4$ WT and KO	CD1/SV129
$P2Y_2$/$P2Y_4$ WT and DKO	B6D2/SV129/CD1

indicating that activation of either one may be responsible for the effect (Ralevic & Burnstock, 1998). In respiratory epithelium, the generation of the $P2Y_2$ receptor knock-out (KO) mouse helped to show that the major luminal P2Y receptor involved in the activation of chloride secretion was the $P2Y_2$ subtype (Cressman *et al.* 1999). Not all epithelia express this receptor subtype luminally but still respond vividly to the addition of luminal UTP. Mouse jejunum displays a luminal UTP-stimulated Cl^- secretion, which was not affected in $P2Y_2$ receptor KO mice (Cressman *et al.* 1999; Robaye *et al.* 2003). Using $P2Y_4$ KO mouse it was recently established that the $P2Y_4$ receptor is responsible for the UTP-stimulated Cl^- secretion. Thus, the use of P2 receptor knock-out mice has become a powerful tool to investigate which receptors are expressed in a given tissue (Dubyak, 2003). In the present study we use this strategy to investigate the effect of luminal nucleotides on ion transport in mouse distal colon. Specifically, we address the question, Do luminal UTP and ATP stimulate K^+ secretion as shown previously in rat distal colonic mucosa (Kerstan *et al.* 1998)? In this study we show that both luminal $P2Y_2$ and luminal $P2Y_4$ receptors are functionally expressed in mouse distal colon, and that their activation induces a transient K^+ secretion.

Methods

Mouse strains

All handling and use of animals complied with Danish animal welfare regulations. In the present study different mouse strains were used (Table 1). Previous studies investigating P2 receptor effects on renal ion transport used in-house-bred NMRI mice (Department of Physiology, University of Freiburg) (Lehrmann *et al.* 2002*a*). In the first series of experiments in this paper also NMRI mice were used (Taconic, Lille Skensved, Denmark). The $P2Y_2$ receptor knock-out mouse was generously provided as KO breeder pairs (on a B6D2 genetic background) by Dr B. H. Koller (University of North Carolina, Chapel Hill, USA). B6D2 $P2Y_2^{-/-}$ mice (original $P2Y_2$ KO) were then crossed with the SV129 mouse strain, generating B6D2/SV129 $P2Y_2^{+/+}$ ($P2Y_2$ WT) and B6D2/SV129 $P2Y_2^{-/-}$ ($P2Y_2$ KO) littermates. The mouse $P2Y_4$ receptor gene is localized on the X-chromosome. CD1/SV129 $P2Y_4^{+/o}$ ($P2Y_4$ WT) and CD1/SV129 $P2Y_4^{-/o}$ ($P2Y_4$ KO) littermates were generated as previously described (Robaye *et al.* 2003).

Generation of $P2Y_2$/$P2Y_4$ double receptor knock-out mice

Double knock-out mice were generated by breeding $P2Y_4^{+/-}$ females with $P2Y_2^{-/-}$ ($P2Y_2$ KO) males. From their offspring, $P2Y_2^{+/-}$/$P2Y_4^{+/-}$ females were selected and further crossed with $P2Y_2^{+/-}$ males in order to generate $P2Y_2^{+/+}$/$P2Y_4^{o/+}$ and $P2Y_2^{-/-}$/$P2Y_4^{o/-}$ male littermates. Throughout this article, wild-type males $P2Y_2^{+/+}$/$P2Y_4^{o/+}$ will be referred to as $P2Y_2$/$P2Y_4$ WT and double knock-out males $P2Y_2^{-/-}$/$P2Y_4^{o/-}$ as $P2Y_2$/$P2Y_4$ DKO.

Genotyping of $P2Y_2$ and $P2Y_4$ receptor knock-out mice

Genotyping was performed from extracted DNA from clipped tails. For the $P2Y_2$ mice genotyping, a triple primer genomic PCR was used. The two different forward primers had the following sequences: 5′-GTCACGCGCACCCTCTACTA-3′ (identifying the WT allele) and 5′-GGGGAACTTCCTGACTAGG-3′ (identifying the inserted neo-cassette in the disrupted allele). The reverse primer for both alleles had the following sequence: 5′-GTCGGGTGCACTGCCTTTCT-3′. For the $P2Y_4$ mouse genotyping two genomic PCRs were used. The wild type allele was detected using the primers 5′-AGTAGAGGTTCCAGTAGAAA-3′ and 5′-GACTCC-TTGCTATTCACAT-3′ while the mutated allele was amplified with the primers 5′-CGAAGTTATATTAA-GGGTTC-3′ and 5′-TAATCGGTCACCCTCA-3′. For the $P2Y_2$/$P2Y_4$ WT and DKO genotyping, the previous primers and methods were used.

Ussing chamber experiments

Mice (age 4–20 weeks) were killed by decapitation. Only the distal 2 cm of mouse colon was used. The muscular layers were gently removed and a piece was mounted in an Ussing Chamber with an aperture of 0.126 cm^2. The two halves of the chamber were perfused continuously by a bubble lift system. The solutions on the two sides were composed as follows (mM): NaCl 120; $NaHCO_3$ 25; K_2HPO_4 1.6; KH_2PO_4 0.4; calcium gluconate 1.3; $MgCl_2$ 1; D-glucose 5, indomethacin (5 μM). The reservoirs were bubbled with carbogen (5% CO_2 and 95% O_2) and kept at 37°C by water jackets. The measurements were performed in 'open-circuit' mode. Transepithelial voltage (V_{te}) was referred to the serosal side and transepithelial resistance (R_{te}) was calculated from the voltage deflections (ΔV_{te}) induced by short current pulses (25 μA, 0.6 s) (Lohrmann *et al.* 1995). These deflections were corrected for those obtained with the empty chamber. The equivalent short circuit current (I_{sc}) was obtained by Ohm's law from V_{te}/R_{te}. The calculated I_{sc} changes were derived from peak values. Initially, tetrodotoxin (TTX, 1 μM) was added to the serosal side to inhibit possible secretory activation

Table 2. Summary of used primer sequences

P2Y receptor	Forward primer 5′–3′	Reverse primer 5′–3′	Expected band size of amplicon (bp)
$P2Y_1$	TCTTGGGCTGTTATGGAT	TCTCAGGGATGTCTTGTG	481
$P2Y_2$	GTCACGCGCACCCTCTACTA	TCGGGTGCACTGCCTTTCTT	548
$P2Y_4$	CTTTGGCTTTCCCTTCTTGA	GTCCGCCCACCTGCTGAT	427
$P2Y_6$	GCCCTGTGCTGGAGACCTTC	CATGGCCCCAGTGACAAACA	226

by the enteric nervous system or other autonomous nerve cells. Subsequently, amiloride (100 μM) was added to the mucosal perfusate to abolish any rheogenic Na^+ absorption. After an equilibration period (30–60 min) UTP or other agonists and antagonists were added to the mucosal side. In the experiments performed on low Na^+ diet mice, the animals were fed a special diet containing (mg kg^{-1}): Na^+ 136; Cl^- 178.5; K^+ 7170 (Altromin, Lage, Germany).

Crypt preparation

Mice (age 4–20 weeks) were killed by decapitation (without anaesthesia). The preparation of colonic crypts was similar to that described by Siemer & Gögelein (1992) and Diener *et al.* (1989). A 4 cm piece of mouse distal colon was everted and rinsed with ice-cold Ca^{2+}-free Ringer-type solution with the following composition (mM): NaCl 127; KCl 5; sodium pyruvate 5; D-glucose 5; 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Hepes) 10; ethylenediaminetetraacetic acid (EDTA) 5; $MgCl_2$ 1. Both ends were tied to obtain a sac preparation. This sac was filled with the same Ca^{2+}-free solution. The sacs were then incubated in the above mentioned solution for 10 min at 37°C. Isolated crypts were obtained by shaking the sacs.

RT-PCR analysis of P2Y receptors in isolated mouse colonic crypts

RT-PCR analysis was used to investigate if specific mRNAs for the mouse $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors are present in mucosal epithelial cells. To this end, total RNA was extracted from approximately 500 isolated colonic crypts. Total RNA was transcribed into cDNA using reverse transcriptase. Primer selection was based on published mouse sequences for the different P2Y receptors (Table 2).

Solutions and chemicals

Tetrodotoxin (TTX) was purchased from Latoxan (Rosans, France). All other chemicals were of the highest grade of purity available and were obtained from Sigma-Aldrich Denmark A/S (Vallensbaek, Denmark) and Merck (Darmstadt, Germany).

Statistics

Data are shown as mean values ± S.E.M. (n), where n refers to the number of mucosal preparations or investigated crypts. Student's paired and unpaired t test was used to compare mean values within one experimental series. A P-value of < 0.05 was accepted as indicating statistical significance.

Results

Luminal ATP and UTP induce a rapid and transient increase of positive equivalent I_{sc} in mouse distal colonic mucosa

Initially, we set out to determine if the response to luminal nucleotides is similar in mouse colonic mucosa to that described for rat tissue (Kerstan *et al.* 1998). The experiments were performed in NMRI mice. Figure 1 shows an original trace from an Ussing chamber experiment in which ATP (100 μM), followed by UTP (100 μM), was applied to the luminal side of the mucosa. The resting transepithelial voltage (V_{te}) was close to −1 mV. In the entire experimental series the mean V_{te} was -1.00 ± 0.17 mV and the mean calculated transepithelial resistance (R_{te}) was $29.7 \pm 2.2\ \Omega\ cm^2$ ($n = 24$). Application of ATP led to a very rapid (within 3 s) and transient change of polarity of the transepithelial voltage from approximately −1 mV to +3 mV (mean peak change of V_{te} from a resting -1.00 ± 0.17 to $+3.11 \pm 0.83$ mV under stimulation, $n = 24$). R_{te} decreased from a prestimulatory value of 29.7 ± 2.2 to $24.1 \pm 1.5\ \Omega\ cm^2$ ($n = 24$). The calculated I_{sc} increase amounted to $148.3 \pm 26.3\ \mu A\ cm^{-2}$. After approximately 1 min, UTP was also added onto the mucosal surface. This induced only a very small deflection of V_{te} (0.43 ± 0.09 mV, $n = 22$). Subsequently, the tissue was gently and thoroughly washed and a second testing of luminal nucleotides was undertaken after approx. 20–30 min, with the application order of the luminal nucleotides reversed. In this second series, luminal UTP stimulated a rapid and transient deflection of V_{te} from a mean value of -0.81 ± 0.23 to 3.11 ± 0.61 mV and a decrease in R_{te} from 29.4 ± 2.4 to $23.5 \pm 2.0\ \Omega\ cm^2$ ($n = 24$). Again, a subsequent addition of in this case luminal ATP was almost without effect (V_{te} change 0.32 ± 0.10 mV, $n = 24$). The concentration response curves for the luminal ATP- and UTP-mediated

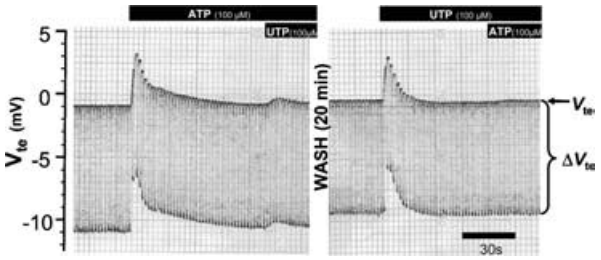

Figure 1. Effect of luminal ATP and UTP on ion transport in mouse distal colonic mucosa (NMRI mice)
Original recording of transepithelial voltage (V_{te}) and transepithelial voltage changes (ΔV_{te}). The upper line shows V_{te} and the bandwidth of voltage deflections reflects the transepithelial resistance (R_{te}, see Methods). Application of luminal ATP or UTP to the luminal side led to a transient deflection of V_{te} to lumen-positive values and a decrease of R_{te}.

effect were investigated in a separate series of experiments in NMRI mice and are shown in Fig. 2. Both agonists were of similar potency. The data were fitted with the Hill equation and the EC_{50} values of 13 μmol l^{-1} for ATP and 10 μmol l^{-1} for UTP were calculated. Neither luminal adenosine nor UDP produced any effects (data not shown). This pharmacological profile strongly suggests that either activation of a $P2Y_2$ or a $P2Y_4$ receptor underlies this effect.

The UTP and ATP effects are inhibited by luminal Ba^{2+} and iberiotoxin

A change of V_{te} to lumen-positive values with a concomitant decrease in R_{te} may, in principle, be the result of either luminal hyperpolarizing conductances or basolateral depolarizing conductances. A luminal hyperpolarization could result from K^+ or Cl^- channel opening in the luminal membrane or a basolateral depolarization

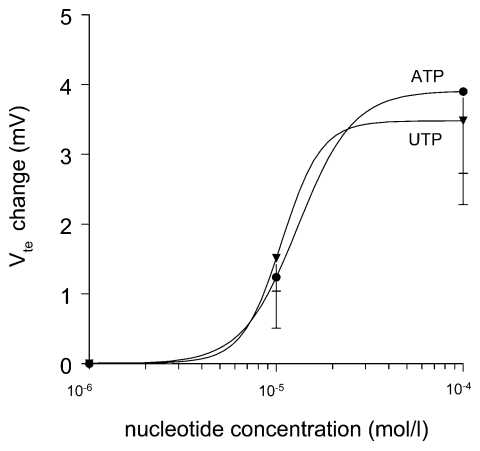

Figure 2. Concentration response relationship of luminal UTP- and ATP-induced transepithelial voltage deflections
Both nucleotides showed a similar potency with an EC_{50} value of 13 μM for ATP and 10 μM for UTP.

from opening of cationic or Cl^- channels in the basolateral membrane. The following experiments were performed in NMRI mice to elucidate which ion conductances were activated by luminal ATP. Extrapolating from our previous rat data (Kerstan *et al.* 1998), it can be assumed that a luminal K^+ channel was responsible for the observed effect. In strictly paired experiments shown in Fig. 3, the ATP (100 μM) effect was investigated in the presence and absence of luminal Ba^{2+} (5 mM) in NMRI mice. Both V_{te} and R_{te} effects of luminal ATP were almost completely blocked by luminal Ba^{2+} (voltage change with Ba^{2+}, 0.38 ± 0.33 mV; voltage change after Ba^{2+} wash-out, 2.84 ± 0.58 mV; $n = 5$). Similar results were obtained when UTP was used as luminal agonist (data not shown). Importantly, luminal iberiotoxin (IBTX, 240 nM) also completely inhibited the UTP-stimulated V_{te} deflection (voltage change without IBTX, 5.25 ± 1.25 mV; voltage change with IBTX, 0.83 ± 0.36 mV; $n = 6$). These results strongly indicate that luminal UTP and ATP activate a luminal BK channel and thus K^+ secretion.

PCR analysis reveals the presence of different P2Y receptors in isolated rat colonic crypts

The above-described data strongly indicated that luminal ATP and UTP mediate their effect via $P2Y_2$ or $P2Y_4$ receptors. To investigate this further, the presence of specific $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor mRNA in mouse colonic crypts of NMRI mice was determined. As shown in Fig. 4, specific P2Y receptor mRNA transcripts were found for the $P2Y_2$, $P2Y_4$ and the $P2Y_6$ receptors (for details see Methods). Similar results were obtained in three different RNA extractions from isolated mouse colonic crypts. These results are consistent with the hypothesis

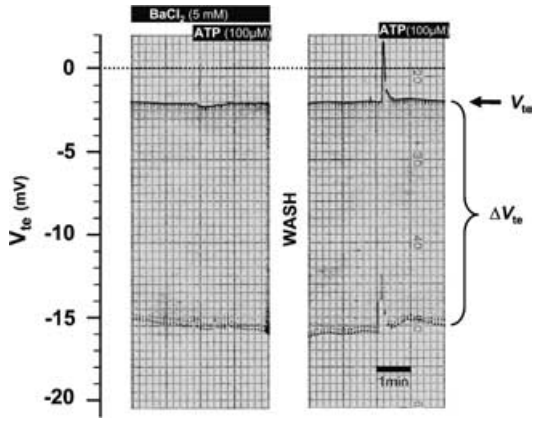

Figure 3. Effect of luminal Ba^{2+} on luminal ATP-induced transepithelial voltage effect in mouse distal colonic mucosa
Original recording of transepithelial voltage (V_{te}) and transepithelial voltage changes (ΔV_{te}). The upper line shows V_{te} and the bandwidth of voltage deflections reflects the transepithelial resistance (R_{te}). Luminal Ba^{2+} completely inhibited the ATP-induced V_{te} deflections. After wash-out this effect was fully reconstituted.

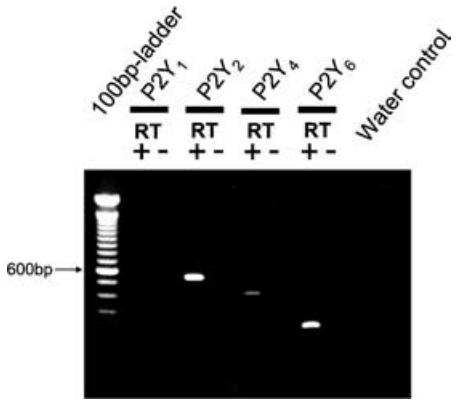

Figure 4. RT-PCR identification of different P2Y receptors mRNA transcripts from isolated distal colonic crypts of NMRI mice
Positive results were found for the $P2Y_2$, the $P2Y_4$ and the $P2Y_6$ receptors.

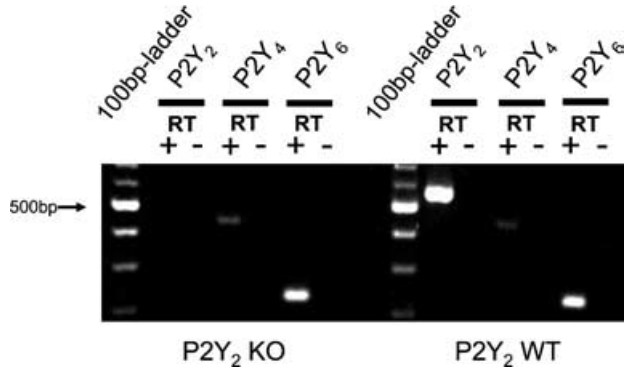

Figure 6. RT-PCR identification of different mouse P2Y receptors mRNA transcripts from isolated distal colonic crypts from $P2Y_2$ KO and WT mice
Positive results were found for the $P2Y_4$ and the $P2Y_6$ receptors in WT and KO. $P2Y_2$ receptor transcripts were absent in the KO mouse and present in the WT mouse.

that either a $P2Y_2$ or a $P2Y_4$ receptor mediates the effects observed in the Ussing chamber.

Reduced K^+ secretory response in $P2Y_2$ receptor KO mice

To explore this in detail, the recently generated $P2Y_2$ receptor KO (original $P2Y_2$ KO, genetic background: B6D2) mouse was used. Breeder pairs of these mice were set to reproduction. Mice of either sex were investigated at the same age as the NMRI mice. In the population of original $P2Y_2$ KO mice, all animals responded with a pronounced response to luminal ATP and UTP. Application of ATP led to a rapid and transient change of polarity of the transepithelial voltage from a resting value of -0.30 ± 0.49 mV to $+2.85 \pm 1.11$ mV under stimulation. R_{te} decreased from a prestimulatory value of $41.3 \pm 5.4\ \Omega\,cm^2$ to $37.8 \pm 4.4\ \Omega\,cm^2$ ($n = 9$). Similarly, the application of luminal UTP led to a rapid and transient change of polarity of the transepithelial voltage from a resting value of -0.35 ± 0.28 mV to $+4.16 \pm 1.04$ mV under stimulation. R_{te} decreased from a prestimulatory value of $46.2 \pm 5.6\ \Omega\,cm^2$ to $38.4 \pm 3.6\ \Omega\,cm^2$ ($n = 10$). These results are similar to those measured in NMRI mice and therefore may indicate that a $P2Y_2$ receptor is not linked to the activation of K^+ secretion. This issue was further tested by comparing $P2Y_2$ receptor KO mice with their littermate WT controls. This was done by crossing the original B6D2 $P2Y_2$ KO mice with SV129 WT mice. The F_2 generation (genetic background: B6D2/SV129) was used for functional experiments. The Ussing chamber results of these experiments are shown in Fig. 5. There is a clear difference between $P2Y_2$ KO mice and their corresponding WT littermates. In WT and $P2Y_2$ KO mice, UTP led to a rapid deflection by 2.08 ± 0.52 mV ($n = 9$) and 0.83 ± 0.26 mV ($n = 11$), respectively. In each $P2Y_2$ receptor KO mouse, luminal UTP (100 μM) induced a smaller but still significant effect. These data strongly suggest that the $P2Y_2$ receptor mediates part of the luminal nucleotide-mediated K^+ secretory response, but yet another receptor may be present to account for the remaining part. We subsequently returned to the RT-PCR analysis and questioned which of the UTP- or UDP-sensitive P2Y receptors are detectable in $P2Y_2$ KO mice (genetic background: B6D2/SV129). The representative gel in Fig. 6 shows no evidence for $P2Y_2$ receptor-specific mRNA, but shows that the $P2Y_4$ and the $P2Y_6$ receptor mRNAs continue to be present. These results

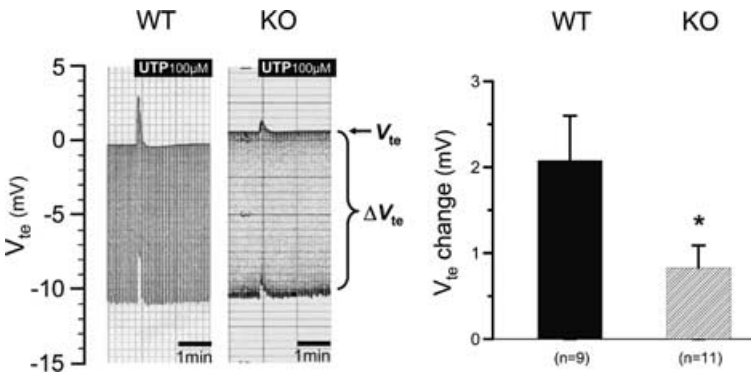

Figure 5. Effect of luminal UTP in $P2Y_2$ receptor KO and WT littermates
Two original recordings of transepithelial voltage (V_{te}) and transepithelial voltage changes (ΔV_{te}) in KO and WT mice. The upper line shows V_{te} and the bandwidth of voltage deflections reflects the transepithelial resistance (R_{te}). The right panel shows the summary of all experiments.

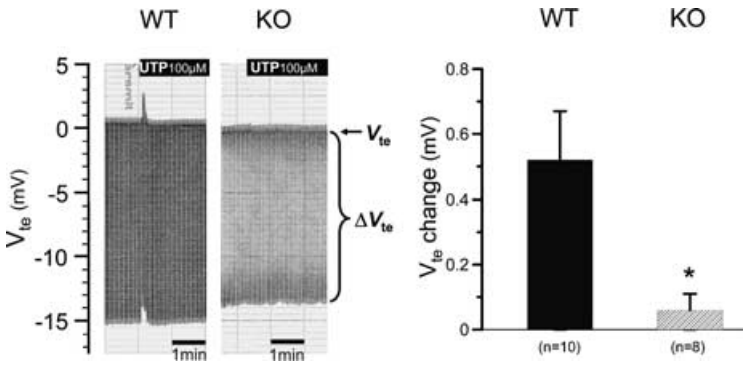

Figure 7. Effect of luminal UTP in $P2Y_4$ receptor knock-out mice in comparison to wild-type littermates
Two original recordings of transepithelial voltage (V_{te}) and transepithelial voltage changes (ΔV_{te}) in KO and WT mice. The upper line shows V_{te} and the bandwidth of voltage deflections reflects the transepithelial resistance (R_{te}). The right panel shows the summary of all experiments.

support the hypothesis that the $P2Y_4$ receptor mediates the residual K^+ secretory effect.

Reduced K^+ secretory response in $P2Y_4$ receptor KO mice

Obviously, the above-mentioned results imply that the $P2Y_4$ receptor may also be expressed in the luminal membrane of distal mouse colonic mucosa. The recently generated $P2Y_4$ KO mice (genetic background: CD1/SV129) (Robaye *et al.* 2003) were used for these experiments following the same approach as for the $P2Y_2$ KO mice. In $P2Y_4$ WT littermates, luminal UTP induced the well described rapid and transient change of polarity of the V_{te} from a resting value of -0.47 ± 0.25 mV to $+0.06 \pm 0.33$ mV ($n = 10$) (Fig. 7). However, the magnitude of the UTP-induced V_{te} deflection was significantly smaller compared to both the NMRI mice (Figs 1 and 3) and the $P2Y_2$ WT mouse (Fig. 5). Luminal UTP showed no effect in $P2Y_4$ KO mice (resting -0.21 ± 0.18 mV to -0.15 ± 0.17 mV, $n = 8$). In one single experiment in a $P2Y_4$ KO mouse, a very small V_{te} deflection of 0.40 mV was observed. These results support the hypothesis that the luminal membrane also expresses $P2Y_4$ receptors. However, if both receptors are expressed in the luminal membrane one would expect a residual effect of luminal UTP in the $P2Y_4$ KO mice. Figure 8 shows the RT-PCR results from isolated colonic crypts of a $P2Y_4$ KO mouse (genetic background: CD1/SV129) indicating the absence of $P2Y_4$ transcripts but the presence of $P2Y_2$ and $P2Y_6$ mRNA transcripts. Thus, the $P2Y_2$ receptor continues to be expressed in the $P2Y_4$ KO mouse.

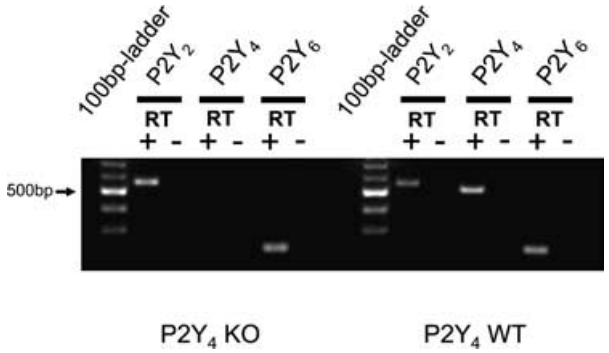

Figure 8. RT-PCR identification of different mouse P2Y receptor mRNA transcripts from isolated distal colonic crypts of $P2Y_4$ KO and WT mice
Positive results were found for the $P2Y_2$ and the $P2Y_6$ receptors in WT and KO. $P2Y_4$ receptor transcripts were absent in the KO and present in the WT mice.

In the case of $P2Y_4$ receptor WT mice, the nucleotide-induced effect is very small and may not allow us to observe any potential residual $P2Y_2$ receptor-mediated response in the $P2Y_4$ KO littermates. To address this issue, the conditions were manipulated in order to see an augmented luminal nucleotide-stimulated response. Recently, the BK channel was identified as the channel responsible for the nucleotide-induced K^+ secretion (Leipziger *et al.* 2003). BK channels are activated both by depolarization and increases in $[Ca^{2+}]_i$. Depolarization of the cells reduces the level of $[Ca^{2+}]_i$ needed to activate BK channels (Kanazirska *et al.* 1995). A low Na^+ diet for 2 weeks enhances the ENaC-mediated electrogenic Na^+ absorption in an aldosterone-dependent fashion, and thereby significantly depolarizes the luminal membrane of mouse distal colon (Will *et al.* 1985). Figure 9 shows two original Ussing chamber traces of mouse distal colonic mucosa from a $P2Y_4$ WT and a corresponding $P2Y_4$ KO littermate (genetic background: CD1/SV129). Electrogenic Na^+ absorption under these conditions is greatly increased as seen by the large lumen-negative V_{te} close to -15 mV in both traces. In the entire series of experiments, this augmented amiloride-inhibitable Na^+ absorption amounted to 657.0 ± 118.8 μA cm^{-2} ($n = 10$) in $P2Y_4$ WT mice and to 667.1 ± 181.3 μA cm^{-2} ($n = 11$) in $P2Y_4$ KO mice. Under these conditions, the luminal UTP-stimulated V_{te} deflection in $P2Y_4$ WT mice was significantly increased to 2.37 ± 0.34 mV, as compared to mucosa from animals on a normal diet. Importantly, all $P2Y_4$ KO mice now responded with a K^+ secretory response (ΔV_{te} change: 1.17 ± 0.33 mV) upon nucleotide stimulation. Figure 10 summarizes the results of all experimental series. Interestingly, in all investigated mice ($P2Y_2$ WT and KO, $P2Y_4$ WT and KO), the K^+ secretory response was significantly increased in animals on a low

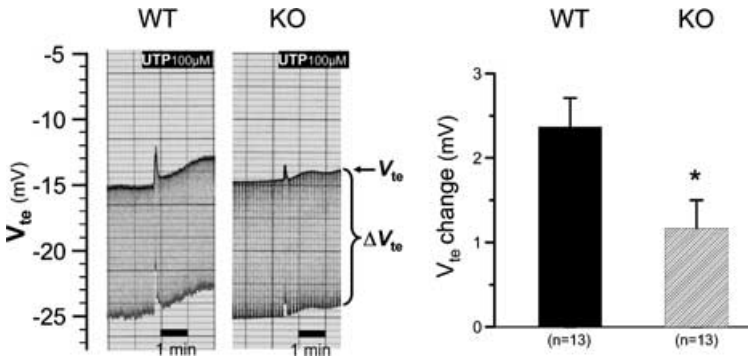

Figure 9. Effect of luminal UTP in $P2Y_4$ receptor knock-out mice in comparison to wild-type littermates treated on a low Na^+ diet for 2 weeks
Two original recordings of transepithelial voltage (V_{te}) and transepithelial voltage changes (ΔV_{te}) in KO and WT mice. The upper line shows V_{te} and the bandwidth of voltage deflections reflects the transepithelial resistance (R_{te}). Note the significantly more lumen negative V_{te} values in both original traces as compared to those in Figs 1, 3, 5 and 7. Importantly, in all $P2Y_4$ KO mice, luminal UTP always induced the rapid and transient V_{te} deflection. The right panel shows the summary of 13 mouse pairs.

Na^+ diet, supporting the hypothesis that a low Na^+ diet, in general, facilitates luminal nucleotide stimulated BK-dependent K^+ secretion.

Furthermore, these results indicate that in mice lacking $P2Y_4$ receptors, a significant fraction of the luminal UTP-stimulated K^+ secretory response is reduced, and that a significant residual UTP-mediated K^+ secretion remains. This remaining K^+ secretion is likely to be mediated via a luminal $P2Y_2$ receptor. In summary, these results are consistent with the interpretation that both a luminal $P2Y_2$ and a luminal $P2Y_4$ receptor are linked to the activation of a transient K^+ secretion.

Abolished UTP-stimulated K^+ secretory response in $P2Y_2/P2Y_4$ double receptor knock-out mice

To investigate the above-stated hypothesis we generated $P2Y_2/P2Y_4$ double receptor KO mice (DKO) (genetic background: B6D2/SV129/CD1) (see Methods). Upon simple inspection, $P2Y_2/P2Y_4$ DKO mice showed no gross abnormalities. Male $P2Y_2/P2Y_4$ WT and DKO mice were investigated at the age of 4–20 weeks. As shown above, the luminal nucleotide-stimulated K^+ secretory response is significantly up-regulated in mice treated on a low Na^+ diet. Therefore, these experiments were conducted after the animals had received a low Na^+ diet for 21 days. In the population of $P2Y_2/P2Y_4$ WT, all animals responded with a pronounced response to luminal UTP (Fig. 11). Application of UTP led to a rapid and transient change of the transepithelial voltage from a prestimulatory value of -8.76 ± 1.40 mV to -4.16 ± 1.25 mV under peak stimulation ($n = 7$). In sharp contrast, addition of luminal UTP to the colonic mucosa of $P2Y_2/P2Y_4$ DKO mice showed no effect (ΔV_{te}: 0.00 ± 0.05 mV, $n = 7$). These results prove that both $P2Y_2$ and $P2Y_4$ receptors are expressed in the luminal membrane of mouse distal colonic mucosa and mediate the luminal nucleotide-stimulated K^+ secretion.

No effect of basolateral UTP on transepithelial ion transport in mouse distal colonic mucosa

Basolateral nucleotides have previously been shown to regulate ion transport in the intestine. For example, basolateral ADP and ATP stimulate NaCl secretion via a $P2Y_1$ receptor and basolateral UDP stimulated NaCl secretion via a $P2Y_6$ receptor (Leipziger *et al.* 1997; Köttgen *et al.* 2003). Here, the effect of basolateral UTP on mouse distal

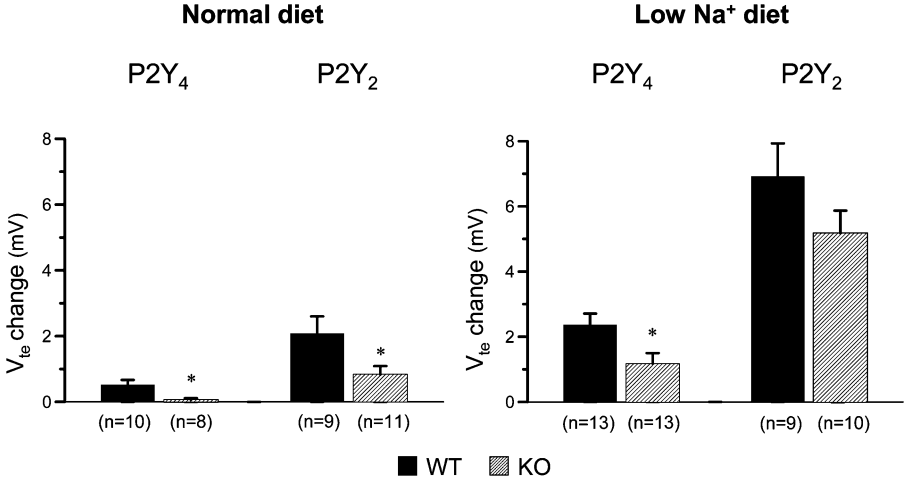

Figure 10. Summary of all UTP-stimulated K^+ secretory effects (rapid and transient V_{te} deflections) in $P2Y_2$ and $P2Y_4$ WT and KO mice on normal and low Na^+ diet
Note the significant up-regulation of this effect in all tested animals after the treatment on a low Na^+ diet.

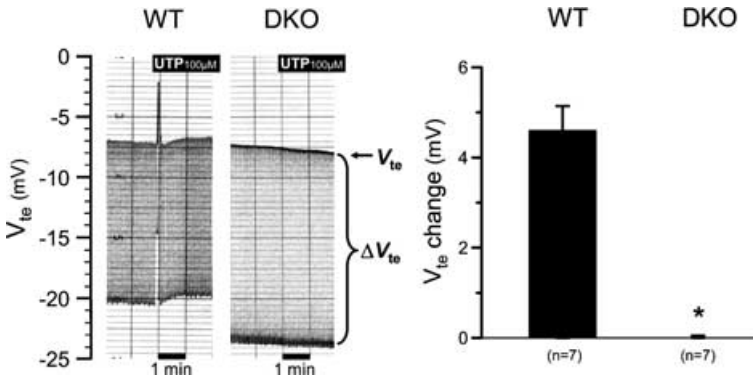

Figure 11. Absence of luminal UTP-stimulated K^+ secretion in $P2Y_2/P2Y_4$ double receptor KO mice in comparison to wild-type littermates
Two original recordings of transepithelial voltage (V_{te}) and transepithelial voltage changes (ΔV_{te}) in DKO and WT mice. The upper line shows V_{te} and the bandwidth of voltage deflections reflects the transepithelial resistance (R_{te}). The right panel shows the summary of 7 mouse pairs.

colon was tested (genetic background: CD1/SV129). A representative trace from a $P2Y_4$ WT is shown in Fig. 12. Basolateral UTP stimulated only a minimal deflection to more lumen-negative V_{te} values. In the entire series, V_{te} changed by 0.06 ± 0.04 mV to more lumen negative values upon stimulation with basolateral UTP. Only two out of five experiments showed this very small effect. Similarly, basolateral UTP in the $P2Y_2$ WT mouse was ineffective (V_{te} change 0.34 ± 0.24 mV, $n = 5$). The subsequent addition of forskolin shows the well-known effect on Cl^- secretion with a large change of V_{te} to lumen-negative values. These results indicate that basolateral UTP does not stimulate the observed K^+ secretion induced by the addition of UTP to the luminal side.

Discussion

K^+ secretion via $P2Y_2$ and $P2Y_4$ receptors

Epithelia commonly respond to luminal nucleotides, such as ATP and UTP, modifying ion transport processes (Leipziger, 2003). UTP specifically activates the so-called 'pyrimidine' receptors $P2Y_2$ and $P2Y_4$ (Ralevic & Burnstock, 1998). It remains a difficult task to unravel which of these receptors actually mediates a given response, since specific antagonists are pending for these receptors (Ralevic & Burnstock, 1998). Therefore, the pharmacological profile regarding different agonists has been applied to differentiate these receptors. It was found that the human $P2Y_2$ receptor is equally activated by ATP and UTP and not by ADP or UDP. In contrast, the human $P2Y_4$ receptor displays a preference for UTP, with ATP being two orders of magnitude less potent (Nichols *et al.* 1996; Kennedy *et al.* 2000). In an earlier study in rat colonic mucosa, it was found that UTP and ATP stimulated a transient K^+ secretion with similar potency (Kerstan *et al.* 1998). We assumed that a luminal $P2Y_2$ receptor mediated this K^+ secretion. Shortly after, however, it was recognized that the discriminative pharmacological profile for the human $P2Y_2$ and $P2Y_4$ receptors does not apply for rodent orthologues, as they were shown to be similarly activated by UTP and ATP (Bogdanov *et al.* 1998; Kennedy *et al.* 2000). Therefore, the question which of the receptors mediates distal colonic K^+ secretion remained unanswered. A similar conflict of receptor identification was recognized in the gerbil stria vascularis of the inner ear. In an initial study, the authors identified a P2U receptor (later renamed $P2Y_2$ (Fredholm *et al.* 1997)) responsible for inhibition of K^+ secretion, similarly activated by ATP and UTP (Liu *et al.* 1995; Marcus *et al.* 1997; Marcus & Scofield, 2001). Nonetheless, it must be appreciated that any previously described P2U receptor-mediated effect in rodents may reflect either the $P2Y_2$ and/or the $P2Y_4$ receptor. Recent immunohistochemical and functional data suggest that, in the inner ear stria marginal cells and vestibular dark cells, the $P2Y_4$ receptor leads to the observed effect (Marcus & Scofield, 2001; Sage & Marcus, 2002).

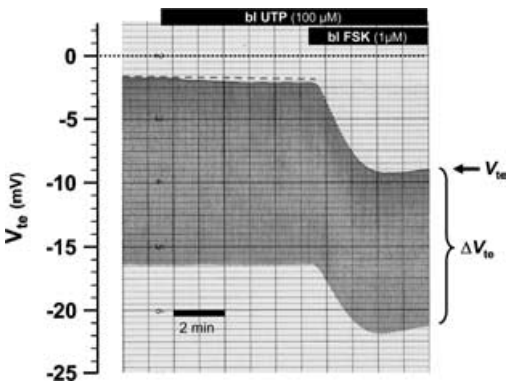

Figure 12. No effect of basolateral UTP in a $P2Y_4$ receptor WT mouse
An original recording of transepithelial voltage (V_{te}) and transepithelial voltage changes (ΔV_{te}). The upper line shows V_{te} and the bandwidth of voltage deflections reflects the transepithelial resistance (R_{te}). The dashed line represents the baseline before addition of basolateral UTP.

In airway epithelium, results from the $P2Y_2$ KO mouse led to the identification of a luminal $P2Y_2$ receptor important for nucleotide-induced Cl^- secretion (Cressman *et al.* 1999). This study has also shown that the $P2Y_2$ receptor is not involved in the luminal stimulation of Cl^- secretion in other tissues, like the small intestine. A recent publication using a $P2Y_4$ KO mouse identified

a luminal $P2Y_4$ receptor as responsible for UTP-activated Cl^- secretion in the small intestine (Robaye *et al.* 2003). This study is the first to show a clear-cut biological effect of the $P2Y_4$ receptor.

In our study, we show that luminal UTP/ATP activates a prompt and transient opening of luminal K^+ channels in mouse distal colon, similar to that shown in rat. The observed V_{te} deflection is caused by opening of luminal K^+ channels because the effect is luminal Ba^{2+} and iberiotoxin sensitive.

In a preliminary account, we have shown that the luminal nucleotide-stimulated K^+ secretion is mediated via BK channels (Leipziger *et al.* 2003). In the present study, the use of $P2Y_2$ and $P2Y_4$ KO mice reveals that both $P2Y_2$ and $P2Y_4$ receptors mediate this K^+ secretion. This conclusion is based on the finding that a significant part of the nucleotide-induced K^+ secretion remained in $P2Y_2$ KO mice. This residual component is therefore likely to be mediated via the other UTP receptor ($P2Y_4$). Indeed, this K^+ secretion was absent in $P2Y_4$ receptor KO mice. These results are interesting and support the notion that the $P2Y_4$ receptor is also expressed in the luminal membrane of mouse distal colon. However, the absence of a UTP effect in $P2Y_4$ KO mice is puzzling. If both receptors are present, one would expect a remaining UTP effect in the $P2Y_4$ KO mouse. The small size of the K^+ secretory response in the $P2Y_4$ WT may have hindered the detection of a potential $P2Y_2$ receptor-mediated effect in the KO littermate. It is noteworthy that in one out of eight experiments, we did observe a very small V_{te} deflection in a $P2Y_4$ KO mouse.

The magnitude of the V_{te} deflection varied between the different mouse strains. The UTP-induced V_{te} deflection was 3.92 ± 0.61 mV in NMRI mice and 4.51 ± 1.03 mV in B6D2 mice. However, in the B6D2/SV129 ($P2Y_2$) strain, the V_{te} effect was smaller (2.08 ± 0.52 mV). In the CD1/129 ($P2Y_4$) strain, the V_{te} effect was even smaller amounting to 0.52 ± 0.15 mV. The reason for this difference is unknown, but the genetic background of the different mice may give rise to the variability of this phenotype. The magnitude of the V_{te} signal is likely to be quantitatively correlated to the different elements involved in this signal transduction. These may include the amount of luminal $P2Y_2$ or $P2Y_4$ receptor proteins, the magnitude of the subapical intracellular Ca^{2+} signal or the density of BK channels. Further work will be needed to define this issue. We then tried to find conditions in which the luminal UTP/ATP-stimulated K^+ secretion could be augmented. Since BK channels are both Ca^{2+} and depolarization activated, we searched for a way to depolarize the luminal membrane. This was successfully achieved by putting the mice on a low Na^+ diet which will increase distal colonic ENaC-mediated Na^+ absorption (Will *et al.* 1985) inducing a depolarization of the luminal membrane. Under these conditions the luminal UTP-stimulated K^+ secretion was significantly augmented in all the tested mice, i.e. $P2Y_4$ WT and KO and $P2Y_2$ WT and KO. The most important finding is that a residual UTP-stimulated K^+ secretion was always present in $P2Y_4$ KO mice. This is consistent with the hypothesis that the $P2Y_2$ receptor mediates the remaining effect. The absence of the luminal UTP-stimulated K^+ secretion in $P2Y_2/P2Y_4$ DKO mice, in our opinion, proves that both receptors are expressed in the luminal membrane of mouse distal colonic mucosa.

Luminal localization of $P2Y_2$ and $P2Y_4$ receptors

We propose that both $P2Y_2$ and $P2Y_4$ receptors are localized in the luminal membrane of distal colonic mucosa. This is based on the observation that only luminal addition of nucleotides elicits K^+ secretion, together with the rapid nature of the V_{te} deflection. Basolateral UTP had no effect. A potential leak of luminally applied agonist to the basolateral side and subsequent activation of basolateral P2Y receptors would be expected to elicit an effect with a significant delayed time course. Intriguingly, a basolateral $[Ca^{2+}]_i$ elevating agonist would be expected to stimulate the basolateral SK4 channel, resulting in a more lumen-negative V_{te}. This was not observed. The tight distal epithelium of distal colon represents a significant diffusion barrier.

Nonetheless, immuno-histochemical proof is pending. Our own attempts to localize the $P2Y_2$ receptor were unsuccessful. These findings are the first indication that the $P2Y_2$ and the $P2Y_4$ receptors are localized in the same luminal membrane of an epithelial preparation. In this study, the question of basolateral localization of the different P2Y receptors is not addressed.

The transient nature of the luminal nucleotide-stimulated K^+ secretion

In the present study, a significant variation of the luminal nucleotide-mediated V_{te} deflection was observed. It is noteworthy that the effect is transient. A similar case was observed in rat tissue (Kerstan *et al.* 1998). The question at the time was whether a rapid breakdown of the nucleotide could underlie the fast deactivating time course. For that purpose, a continuously perfused Ussing chamber, assuring stable agonist concentrations, was utilized. The time course of the effect was not changed by this manoeuvre and thus it was concluded that a rapid 'desensitization' of the mechanism occurred. Interestingly, a recent study investigated the molecular prerequisites for $P2Y_4$ receptor desensitization and identified three distinct C-terminal serine residues important for internalization of the receptor (Brinson & Harden, 2001). These results were compared to the $P2Y_6$ receptor, which has a slower desensitization kinetic and lacks the relevant C-terminal end (Robaye *et al.* 1997; Brinson & Harden, 2001). It is speculated that the transient nature of this secretory response is due to a specific desensitization feature of the

$P2Y_4$ and the $P2Y_2$ receptors. Although not investigated explicitly in this study, 're-sensitization' of the effect normally occurred after 30–45 min (wash of the Ussing chamber and 30 min equilibration). Another important aspect of the transient nature of this response is likely to be related to the assumed local nucleotide-stimulated $[Ca^{2+}]_i$ transient. We speculate that this $[Ca^{2+}]_i$ transient has a similar time course to that observed in the V_{te} signal.

Other regulated ion conductances by luminal nucleotides

Inspection of Fig. 9 indicates that in addition to the described UTP-stimulated K^+ secretion, other ion transport processes are regulated. It is apparent that shortly after the V_{te} peak a secondary decrease of V_{te} arises. This is due to the inhibition of electrogenic Na^+ absorption and not discussed here (Lehrmann *et al.* 2002*a*). One may also question if luminal nucleotides are able to activate CFTR-mediated Cl^- secretion, as shown for rat jejunum (Robaye *et al.* 2003). In our data, a major activation of Cl^- secretion was not apparent. Inhibition of K^+ secretion with Ba^{2+} or iberiotoxin did not unmask Cl^- secretion. It is puzzling as to why the jejunum and the distal colon should behave differently. We speculate that the answer is hidden in the difference of experimental conditions of the two studies. In our study, we used basolateral TTX (1 μM) in order to reduce any pro-secretory effects, which may occur via the enteric nervous system or other neuronal elements intrinsic to the gut wall. Thus, our conditions favour a more complete deactivation of Cl^- secretion in which CFTR, in a bottleneck-like fashion, determines Cl^- secretion. In the intact mammalian colon, CFTR is the exclusive luminal Cl^- exit pathway and needs preactivation before manoeuvres known to increase the driving force, i.e. opening of K^+ channels, can stimulate Cl^- secretion (Greger *et al.* 1997). This is nicely illustrated in a study on T84 cells (Stutts *et al.* 1995) or in data from CF KO mice (Colledge *et al.* 1995). It is possible that in the study on jejunum some CFTR preactivation was present to allow for luminal UTP-mediated Cl^- secretion via an increase of K^+ channel-dependent driving force.

Physiological role of luminal P2 receptor-mediated ion transport

To understand the physiological relevance of luminal P2 receptors, the question of a natural or pathological source for extracellular nucleotides has to be addressed. Either the extracellular nucleotide could originate from an external source (e.g. colonic bacteria) or could be released from the epithelium itself. Evidence for a bacterial source in the gut is pending. Interestingly, a recent study showed that enteropathogenic *E. coli* triggered the release of ATP from different cell lines including the colonic T84 cell line. Subsequently, the released ATP was broken down to adenosine, which stimulated chloride secretion via A2b receptors on the luminal side of T84 cells (Crane *et al.* 2002). The authors suggest that the *E. coli* stimulated release of nucleotides could account for the enteropathogen-induced diarrhoea. The common denominator for secretory epithelia like the airways, the conjunctiva of eye or the small and large intestine is that activation of luminal P2 receptors can stimulate Cl^-, K^+ or HCO_3^- secretion or inhibit Na^+ absorption, resulting in an increase in the amount of fluid on the luminal surface of the epithelium. This mechanism could therefore serve to remove noxious particles by flushing away a damaging environment. Luminal nucleotides have consequently been implicated as elements of an innate defense mechanism of outer epithelial surfaces (Lazarowski & Boucher, 2001; Leipziger, 2003).

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