Regulation of transepithelial ion transport by two different purinoceptors in the apical membrane of canine kidney (MDCK) cells

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1 The effect of extracellular nucleotides on the transepithelial ion transport of Madin Darby canine kidney cells (MDCK) was investigated. Cells were grown up to confluency on permeable supports and the short circuit current (I_{sc}) was measured with an Ussing chamber-like mini-perfusion system.

2 Apical ATP stimulated a biphasic I_{sc} increase consisting of a first rapid and transient peak followed by a broader one.

3 The first peak evoked by ATP was reversibly blocked by basilen blue (BB) in a concentration-dependent fashion, with an EC_{50} of 7.5 μ M.

4 The P_{2Y} receptor agonist, 2-methylthioATP (2-MeSATP) caused a single transient I_{sc} increase that was completely blocked by pretreatment with BB. On the contrary, the P_{2X} agonist, α,β -methylene ATP (α,β -meATP) was almost completely ineffective on I_{sc} . UTP essentially induced a monophasic response the time-course of which resembled that of the second peak stimulated by ATP. The agonist potency order was 2-MeSATP $\geq$ ATP $\gg$ UTP, α,β -meATP for the first peak and UTP $\geq$ ATP $>$ 2-MeSATP $>$ α,β -meATP for the second peak.

5 Monolayer incubation with the membrane permeable calcium chelator [*bis-o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid, tetra(acetoximethyl)-ester] (BAPTA/AM) inhibited the ATP-evoked first peak.

6 The non-hydrolyzable ATP analogue, adenosine-5'-*O*-(3-thio)-triphosphate (ATP- γ -S) elicited a biphasic response similar to that of ATP. The P_1 receptor agonist, 2-chloroadenosine and CGS-21680, were almost unable to induce an I_{sc} increase. These results rule out the involvement of ATP hydrolysis and P_1 receptor activation as responsible for I_{sc} increase.

7 Inhibition of prostaglandins synthesis by indomethacin abolished the second ATP-evoked peak.

8 Chloride replacement with gluconate on both sides of the epithelium completely inhibited the second peak induced by ATP but only reduced the amplitude of the first spike.

9 The results suggest that ATP stimulates I_{sc} increase by two mechanisms. The first one is mediated by a P_{2Y} receptor and by intracellular calcium increase. The second induces prostaglandin synthesis probably through a P_{2U} receptor activation.

Keywords: MDCK cells; ATP; UTP; P_2 purinoceptors; transepithelial ion transport; short circuit current

Introduction

By interacting with specific membrane receptors termed P_2 , extracellular ATP elicits a variety of responses in a large number of cell types (Benham & Tsien, 1987; Okajima *et al.*, 1987; Gerwins & Fredholm, 1992; Rugolo *et al.*, 1993; Galletta *et al.*, 1994). In particular, purinoceptor stimulation results in activation of transepithelial chloride transport in human airway epithelia (Mason *et al.*, 1991), human intestinal T84 cells (Dho *et al.*, 1992), and Madin-Darby canine kidney (MDCK) cells (Simmons, 1981a,b). Although two ATP receptor genes have been cloned recently (Lustig *et al.*, 1993; Webb *et al.*, 1993), the number and characteristics of P_2 purinoceptors are still unclear. Recent classification, based on the potency order of ATP analogues and response to UTP, postulates the presence of at least 5 receptors termed P_{2X} , P_{2Y} , P_{2U} , P_{2T} and P_{2Z} (O'Connor *et al.*, 1991; Watson & Girdlestone, 1994). While the P_{2X} type seems to be a ligand-gated cation channel, P_{2Y} receptors are commonly associated with phospholipase C activation, inositol phosphates cascade and cytosolic free Ca^{2+} increase. P_{2U} receptors seem also to elicit inositol phosphate turnover, but besides ATP they are also activated by UTP for which reason they have been called nucleotide receptors (O'Connor *et al.*, 1991). P_{2Z} purinoceptors seem to have limited distribution since they are

mainly found in mast cells and other immune cells where they form a large membrane channel. P_{2T} purinoceptors are essentially ADP receptors and are found on platelets.

Simmons (1981a,b) reported the ability of extracellular nucleotides to affect transepithelial ion transport in MDCK cells. ATP, ADP, UTP, and ITP applied to the basolateral side were all effective in increasing Cl^- secretion through a mechanism possibly involving prostaglandin synthesis. Simmons (1981a) also mentioned that apical application of ATP was equally effective but, in contrast with the basolateral stimulation, it was characterized by a biphasic response. We investigated whether this feature was due to the presence of two separate transduction mechanisms. The data obtained in this study demonstrate the presence of at least two ATP receptors distinguished by their sensitivity to basilen blue, selectivity to different nucleotides and mechanism of signal transduction.

Methods

Cell culture

MDCK-1 cells were routinely cultured on plastic culture flasks in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 supplemented with 10%

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foetal calf serum, 2 mM L-glutamine, 100 μml^{-1} penicillin, 100 $\mu\text{g ml}^{-1}$ streptomycin. Aliquots of 2×10^6 cells were stored in liquid nitrogen. Experiments were prepared by thawing and seeding cells on Costar's Transwell-COL cell culture chamber inserts (diameter 24.5 mm) at a density of 75,000 cells cm^{-2} . Cells were allowed to grow to confluency with change of medium every 2 days.

Electrical measurements

After 3 to 7 days in culture, Transwell cups were mounted in a modified Ussing chamber (miniperfusion system Trans-24, World Precision Instruments). The transepithelial potential difference was short-circuited with a voltage clamp (Mod. 558-C5, Bioengineering, The University of Iowa) connected to apical and basolateral chambers via Ag/AgCl electrodes. Potential sensing electrodes were connected to the experimental solution through agar bridges containing 150 mM NaCl. The potential difference and the fluid resistance between these electrodes were compensated. Transepithelial resistance was calculated from the ratio of the potential difference measured in open-circuit conditions to the short circuit current. Epithelia with resistances higher than 600 Ωcm^{-2} were considered for analysis. Experiments were performed at room temperature (20–24°C).

Solutions

Where not otherwise specified, the experiments were carried out in a symmetrical solution containing (in mM): NaCl 126, KH_2PO_4 0.38, K_2HPO_4 2.13, MgSO_4 1, CaCl_2 1, NaHCO_3 24, glucose 10 and phenol red 0.04. The solution was continuously bubbled with 5% CO_2 :95% air. In those experiments where the role of intracellular calcium was evaluated, the cell monolayer was incubated for 30 min in the experimental solution containing 20 μM *bis*-(*o*-aminophenoxy)ethane-*N*, *N'*, *N'*, *N'*-tetraacetic acid, tetra (acetoximethyl)-ester (BAPTA/AM). Thereafter the epithelium was washed with BAPTA/AM-free solution and used for electrical measurements.

Statistics

We observed that the magnitude of the ATP-evoked I_{sc} increase displayed some variability between separate batches of MDCK monolayers. Nevertheless, the shape of the response and the ratio of the first to the second peak were constant. Given this variability of current amplitude, already noted by Simmons (1981b), the comparison between different experimental conditions was performed by collecting data from the same cell preparation. Data are presented as raw current measurement or as mean $\pm$ standard error of the mean (s.e.mean). Significance of differences between mean values was tested by Student's two-tailed *t* test. Rank potency orders of the agonists were obtained by comparing the estimated EC_{50} s.

Materials

2-Methylthio ATP (2-MeSATP), α,β -methylene ATP (α,β -meATP), basilen blue (BB), 2-chloroadenosine, and CGS-21680 were purchased from Research Biochemicals International. [*bis*-(*o*-aminophenoxy)ethane-*N*,*N'*,*N'*,*N'*-tetraacetic acid, tetra(acetoximethyl)-ester] (BAPTA/AM) and UTP were from Calbiochem. Adenosine-5'-O-(3-thio)-trisphosphate (ATP- γ -S) was obtained from Boehringer Mannheim. Indomethacin (Liometacen) was from Chiesi Farmaceutici. ATP and other chemicals were from Sigma.

Results

When MDCK monolayers were mounted in the perfusion chamber and voltage clamped, low values of short circuit

current (I_{sc}) were recorded ($0.5 \pm 0.05 \mu\text{A cm}^{-2}$, $n = 127$). The epithelia exhibited a relatively high transepithelial resistance of $3372 \pm 276 \Omega\text{cm}^{-2}$. Addition of ATP (50–1000 μM) to the apical side of MDCK cells elicited a biphasic activation of I_{sc} . As depicted in Figure 1a, a sharp transient peak at 15–30 s was followed by a second smoother response that peaked at about 2 min. For clarity, these two phases of the ATP response will be indicated as the first peak and the second peak throughout the test. ATP concentrations giving 50% of the maximum response (EC_{50}) were 80 μM and 116 μM for the first and the second peak respectively (Figure 2). In the presence of ATP, the I_{sc} did not completely return to basal level even after 20 min. However, a complete recovery was attained by extensively washing the apical chamber with a volume of fresh solution at least ten times that of the chamber. A second application of ATP, 15 to 25 min after the first one, was again effective but the current amplitude of the second peak was reduced to 45% ($n = 5$).

Interestingly, when basilen blue (BB, 100 μM) was administered to the apical side of the epithelium, the first peak evoked by ATP was dramatically reduced (Figure 1a). The inhibitory effect of BB was completely reversible since ATP was able to elicit a large biphasic response when again administered after washing (not shown). Application of BB to the basolateral side of the epithelium did not affect the response to apical ATP ($n = 3$; not shown). The inhibitory effect of apical BB was dose-dependent, higher molar additions resulting in diminished peak response. The EC_{50} of BB on the ATP-evoked first peak was 7.5 μM , as shown in Figure 1b.

Figure 1 (a) Stimulation of I_{sc} by apical ATP in the absence (O, $n = 13$) and in the presence (●, $n = 5$) of 100 μM basilen blue (BB). In contrast with the second peak, the first peak elicited by ATP was significantly inhibited ($P < 0.001$) by BB. (b) Dose-dependent inhibition of the ATP-induced I_{sc} by apical BB. The peak obtained at 15–20 s from the application by 100 μM ATP was plotted against BB concentration. Each individual value was normalized to the mean I_{sc} peak obtained with ATP alone on the same day. Each point is the mean of 4 to 9 experiments. The fit with a Hill plot yields an EC_{50} of 7.5 μM .

Different ATP analogues and nucleotides were tested in the apical solution. The P_{2Y} receptor agonist, 2-MeSATP (O'Connor *et al.*, 1991) at concentrations up to $100\text{ }\mu\text{M}$, evoked a single transient peak that slowly decreased in a monotonic way toward control values (Figure 3a). At a higher concentration ($500\text{ }\mu\text{M}$) 2-MeSATP was also able to induce a second peak. Interestingly, the 2-MeSATP response at $100\text{ }\mu\text{M}$ was completely inhibited by BB (see Figure 3a). Addition of ATP after 2-MeSATP elicited a large delayed response that was devoid of the initial transient peak (Figure 3b).

We also investigated the response of MDCK monolayers to apical UTP (Figures 2 and 4a). This nucleotide induced a slow I_{sc} increase the time-course of which mimicked that of ATP, except that the first peak was very small even at the highest concentrations. In this context, it is worth noting that application of ATP after UTP elicited only a single transient peak (Figure 4b). The P_{2X} agonist α,β -meATP was poorly effective since high concentrations ($> 100\text{ }\mu\text{M}$) produced only minor changes of I_{sc} . Summarising, the agonist potency order was $2\text{-MeSATP} \geq \text{ATP} \gg \text{UTP}, \alpha,\beta\text{-meATP}$ for the first peak and $\text{UTP} \geq \text{ATP} > 2\text{-MeSATP} > \alpha,\beta\text{-meATP}$ for the second peak (see Figure 2).

The possible role of intracellular Ca^{2+} in the ATP-induced I_{sc} increase was investigated by incubating cell monolayers with $20\text{ }\mu\text{M}$ of the membrane permeable Ca^{2+} chelating agent, BAPTA/AM for 30 min before electrical measurements. This treatment significantly inhibited the first peak elicited by ATP but potentiated the second one (Figure 5).

It is well known that membrane ecto-nucleotidases are able to breakdown ATP rapidly to ADP, AMP and adenosine. Adenosine is a ubiquitous modulator acting *via* specific P_1 purinoceptors. In this context, we asked whether part of the

Figure 2 Dose-response relationships of various agonists, ATP (○), UTP (▲), 2-MeSATP (●) and α,β -meATP (□), for the first (a) and the second (b) peak. Each point is the mean of 3 to 7 experiments. EC_{50} for the first peak was 76 and $80\text{ }\mu\text{M}$ for 2-MeSATP and ATP respectively; for the second peak the EC_{50} was 22.5 for UTP and $116\text{ }\mu\text{M}$ for ATP. For abbreviations, see text.

Figure 3 (a) Comparison between the time-course of I_{sc} increase induced by $100\text{ }\mu\text{M}$ ATP (○, $n = 13$), $100\text{ }\mu\text{M}$ 2-MeSATP (●, $n = 6$) and $100\text{ }\mu\text{M}$ BB plus $100\text{ }\mu\text{M}$ 2-MeSATP (■, $n = 3$) applied apically. The first peak evoked by ATP and 2-MeSATP was not statistically different. All data were statistically different from each other from the second minute up to the end of the experiment ($P < 0.05$). (b) Effect of consecutive addition of 2-MeSATP and ATP. The trace is representative of three similar experiments. Vertical and horizontal scale bars represent $5\text{ }\mu\text{A cm}^{-2}$ and 2 min, respectively.

Figure 4 (a) I_{sc} increase evoked by $100\text{ }\mu\text{M}$ apical UTP ($n = 4$). (b) Consecutive addition of UTP and ATP. The trace is representative of three similar experiments. Vertical and horizontal scale bars represent $5\text{ }\mu\text{A cm}^{-2}$ and 2 min respectively.

Figure 5 Time-course of I_{sc} increase induced by 100 μ M apical ATP in control conditions (O, $n = 13$) and after the incubation of the monolayer with 20 μ M BAPTA/AM (■, $n = 6$). The first peak was significantly inhibited ($P < 0.001$) by BAPTA/AM incubation. Values obtained with BAPTA/AM at 4–6 min were significantly higher than those obtained with ATP ($P < 0.05$).

ATP effect could be mediated by P_1 purinoceptors through ATP hydrolysis and rapid accumulation of adenosine. Accordingly, we tested adenosine analogues CGS-21680 and 2-chloroadenosine in the apical chamber. While the former compound was completely ineffective ($n = 3$), the latter caused an I_{sc} increase of less than 0.2 μ A cm^{-2} ($n = 3$, Figure 6). To further test the possibility of adenosine involvement, we also investigated the activity of ATP- γ -S: 100 μ M of this non hydrolyzable ATP analogue elicited a strong I_{sc} increase the time-course of which was comparable to that produced by ATP (Figure 6). The first peak was not statistically different from that obtained with ATP at the same concentration, whereas the more delayed current increment was even higher.

Indomethacin has been found to block fully the response to basolateral nucleotide application in MDCK cells (Simmons, 1981a). We tested the ability of this compound to affect the activity of apical ATP. Addition of 20 μ M indomethacin to both sides of the epithelia 2–6 min before application of ATP completely inhibited the second part of the ATP response (Figure 7) leaving the first peak unaffected. This indomethacin concentration did not significantly modify I_{sc} by itself.

Isosmotic replacement of NaCl with Na-gluconate and of 2 mM $CaCl_2$ with 5 mM $CaSO_4$ completely abolished the second peak stimulated by ATP but only reduced the amplitude of the first spike to 39% of the control ($n = 3$, not shown). Apical addition of the epithelial sodium channel blocker amiloride (100 μ M) did not modify the current amplitude.

Discussion

We have analysed the response of MDCK cells to apical nucleotide stimulation using various agonists and inhibitors. Apical ATP elicited a biphasic I_{sc} response consisting of a first transient spike followed by a second broader current increase. Our study clearly demonstrated that BB is able to inhibit in a dose-dependent fashion the initial peak of the ATP effect. Since BB has been described as an antagonist of P_{2Y} purinoceptors (Burnstock & Warland, 1987), its partial effect in MDCK cells suggests that apical ATP increases I_{sc} by stimulation of two separate receptors one of which belongs to the P_{2Y} group. This seems confirmed by the lack of activity of α, β -meATP and by the fact that 2-MeSATP, a selective agonist of P_{2Y} receptors, at concentrations up to 100 μ M elicited a single transient response that could be completely blocked by BB. Cross desensitization experiments indicated that 2-MeSATP and ATP act on a common receptor, since

Figure 6 I_{sc} increase evoked by 100 μ M apical ATP- γ -S (▲, $n = 4$) and 20 μ M 2-chloroadenosine (□, $n = 3$).

Figure 7 Inhibition of the ATP-evoked I_{sc} increase by indomethacin. Addition of 20 μ M indomethacin 2 to 4 min before application of 100 μ M apical ATP (■, $n = 3$) significantly inhibited ($P < 0.05$) the second peak evoked by ATP in control conditions (O, $n = 13$).

the latter agonist evoked only a monophasic delayed response when applied after the former one. The agonist potency order for the first peak (2-MeSATP $\geq$ ATP $\gg$ UTP, α, β -meATP) is actually different from that of classical P_{2Y} receptors on which 2-MeSATP is sensibly more active than ATP. Nevertheless, the near equipotency of 2-MeSATP and ATP in the present work is similar to that described by Webb *et al.* (1993) for a cloned ATP receptor, blocked by BB and designated as P_{2Y1} . Another interesting observation regarding 2-MeSATP is that it produces a maximum response lower than ATP, suggesting a partial agonist behaviour. The order of potency for the second peak (UTP $>$ ATP $>$ 2-MeSATP $>$ α, β -meATP) is instead consistent with a P_{2U} purinoceptor. Cross-desensitization experiments indicated that UTP and ATP share a common receptor. Indeed, ATP elicited only the first peak when applied following UTP.

Chelation of intracellular Ca^{2+} confirmed that the biphasic response to ATP is due to two different transduction mechanisms. Indeed, BAPTA/AM incubation precluded only the first part of the ATP response. This finding indicates that the putative P_{2Y} receptor in the apical membrane of MDCK cells causes a rapid and transient activation of transepithelial ion transport through an increase in intracellular Ca^{2+} . This could happen *via* phospholipase C stimulation and Ca^{2+} release from intracellular stores, although we cannot exclude an alternative or additive role of extracellular Ca^{2+} influx. The remainder of the ATP response is mediated by a different receptor type and probably by a Ca^{2+} -independent transduction mechanism. We tested whether this response is related to ATP degradation since hydrolysis to adenosine and stimulation of P_1 receptors has been indicated as one of the mechanisms causing ATP-induced Cl^- secretion in T84 cells

(Dho *et al.*, 1992). Nevertheless, this mechanism has been ruled out by our experiments. Adenosine analogues were not able to stimulate significantly I_{sc} . On the other hand, the non hydrolysable ATP- γ -S gave a biphasic effect similar to that of ATP. In addition, UTP, the hydrolysis of which does not produce adenosine, elicited a large response with a time-course resembling that of the second ATP-dependent peak.

The inhibition of the second ATP-evoked peak by indomethacin indicates the involvement of prostaglandins synthesis (Slivka & Insel 1988). Actually, prostaglandins are able *per se* to stimulate transepithelial ion transport in MDCK cells (Simmons, 1991). A mechanism through which prostaglandins can be generated is the Ca^{2+} -dependent activation of phospholipase A_2 . Nevertheless, this possibility seems excluded by the finding that intracellular Ca^{2+} buffering by BAPTA/AM did not prevent the delayed response to ATP. It is possible to speculate that prostaglandin production is independent from P_{2Y} -mediated activation of the phospholipase C pathway. Separate activation of both phospholipase pathways has been indeed demonstrated following stimulation of α_1 -adrenoceptors and bradykinin receptors in MDCK cells (Slivka & Insel, 1987; 1988). Parallel activation of phospholipase A_2 and phospholipase C has also been observed in neutrophils and in HL60 cells stimulated by extracellular ATP (Cockcroft & Stutchfield, 1989).

Another interesting finding regarding the ionic basis of the ATP response was that although the second peak was entirely dependent on the presence of extracellular chloride and therefore consistent with a mechanism of chloride secretion,

the first peak was only partially affected by chloride substitution. This probably indicates the presence of a different mechanism of ion transport the basis of which needs further investigation.

In conclusion, apical ATP stimulates I_{sc} by two different mechanisms. The first one is mediated by a P_{2Y} receptor and by intracellular Ca^{2+} increase. The second is instead mediated by prostaglandins, to some extent is independent of cytosolic calcium and is probably triggered by a P_{2U} purinoceptor, although a conclusive characterization of this receptor is limited by the absence of effective antagonists.

MDCK cells display functional and morphological properties similar to those of the distal tubule and the collecting duct (Valentich, 1981; Herzlinger *et al.*, 1982; Garcia-Perez & Smith, 1983). Our work shows a complex mechanism of regulation of transepithelial ion transport mediated by two purinoceptors in the apical membrane of MDCK cells. This localization poses the important question of the *in vivo* source of luminal nucleotides which should bind to apical receptors. An autocrine mechanism can be postulated through release of ATP and UTP from the same epithelial cells.

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