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Nucleotide-evoked calcium signals and anion secretion in equine cultured epithelia that express apical $P2Y_2$ receptors and pyrimidine nucleotide receptors

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1 Experiments with a spontaneously transformed equine epithelial cell line showed that certain nucleotides increased intracellular free calcium ($[Ca^{2+}]_i$) in cells plated on glass coverslips. The rank order of potency was ATP = UTP > 5-Br-UTP, whilst UDP and ADP were ineffective. The response thus appears to be mediated by $P2Y_2$ receptors.

2 Nucleotides also increased short circuit current (I_{SC}) in cells grown into epithelial monolayers and the rank order of potency was UDP > UTP > 5-Br-UTP > ATP > ADP. The increase in $[Ca^{2+}]_i$ and the rise in I_{SC} thus have different pharmacological properties. Cross-desensitization experiments indicated that, as well as $P2Y_2$ receptors, the monolayer cultures express at least one additional receptor population that allowed nucleotides to increase I_{SC}.

3 The UDP-evoked increase in I_{SC} was essentially abolished in BAPTA-loaded epithelia suggesting that this response is dependent upon increased $[Ca^{2+}]_i$. Moreover, experiments in which I_{SC} and $[Ca^{2+}]_i$ were measured simultaneously showed that the UDP- and ADP-evoked increases in I_{SC} were accompanied by increases in $[Ca^{2+}]_i$.

4 When grown under conditions which favour the development of a polarized phenotype, these epithelial cells thus appear to express $[Ca^{2+}]_i$-mobilizing receptors sensitive to UDP and ADP that are not present in non-polarized cells on coverslips.

Keywords: Pyrimidinoceptors; cell signalling; anion secretion; stimulus-secretion coupling; nucleotide receptors; Ussing chambers

Introduction

Studies of equine epithelial cells (Wilson *et al*., 1993) on glass coverslips or plastic Petri dishes showed that nucleotides increased intracellular free calcium ($[Ca^{2+}]_i$) and raised the ionic permeability of the plasma membrane (Ko *et al*., 1994; Wilson *et al*., 1995; 1996). These effects were attributed to $P2Y_2$ purinoceptors that are activated, with equal efficacy and potency, by adenosine 5′-triposhate (ATP) and uridine-5′-triphosphate (UTP) but which are insensitive to ADP and UDP (Dubyak & El-Moatassim, 1993; Ko *et al*., 1994; Nicholas *et al*., 1996). Subsequent work, in which these cells were grown into functionally polarized epithelia, showed that UTP and ATP also evoked anion secretion, and that the receptors underlying this response were essentially confined to the apical membrane (Ko *et al*., 1997). However, this work also showed that, as well as $P2Y_2$ receptors, the cells expressed 'pyrimidinoceptors' that were sensitive to UTP but not ATP (Ko *et al*., 1997). It is therefore possible that these previously unidentified receptors may have contributed to the $[Ca^{2+}]_i$ signals seen in our earlier study (Ko *et al*., 1994). In the present experiments, we have therefore explored the effects of nucleotides upon the regulation of $[Ca^{2+}]_i$ in non-polarized cells on coverslips and upon the control of anion secretion in polarized epithelial monolayers on permeable supports.

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Methods

Experimental materials

Experiments were undertaken with a spontaneously-transformed cell line derived from the secretory epithelium of the equine sweat gland (Wilson *et al*., 1993). Standard techniques were used to maintain these cells in serial culture; the medium used was serum-free William's medium E supplemented with L-glutamine (1 mM), penicillin (100 iu ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$), bovine insulin (5 $\mu g\ ml^{-1}$), epidermal growth factor (0.1 $\mu g\ ml^{-1}$), hydrocortisone (10 $ng\ ml^{-1}$), transferrin (5 $\mu g\ ml^{-1}$) and sodium selenite (5 $ng\ ml^{-1}$). Bicarbonate-buffered physiological saline contained (in mM): NaCl 117, $NaHCO_3$ 25, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2 $CaCl_2$ 2.5 and D-glucose 11; pH 7.3–7.4 when bubbled with 5% CO_2/95% O_2. The 4-(2-hydroxyethyl)-1-piperazine ethanesulphononic acid (HEPES)-buffered saline contained (in mM): NaCl 120, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, D-glucose 10 and HEPES 20, pH adjusted to 7.4 with NaOH. Cell culture reagents were purchased from Gibco Laboratories (New York, U.S.A.) and general laboratory reagents were from Sigma Chemical Co. (St. Louis, U.S.A.). UDP and ADP were obtained from Sigma and, unless otherwise stated, solutions containing these nucleotides were prepared from 10 mM stock solutions that had been incubated (1 h, 37°C) in HEPES-buffered saline containing hexokinase (10 iu ml^{-1}, Boehringer) and a higher than normal concentration of D-glucose (22 mM). This procedure removes contaminating nucleotide triphosphates (Nicholas *et al*., 1996; Harden *et al*., 1997; Lazarowski *et al*., 1997). Such hexokinase-treated nucleotides were stored at −20°C. UTP and ATP were obtained from Pharmacia as ultrapure solutions. Independent analyses have confirmed that

these nucleotides are essentially pure (R.C. Boucher, personal communication).

Measurement of $[Ca^{2+}]_i$

Cells removed from culture flasks using trypsin/EDTA were washed by centrifugation/resuspension ($\sim 5 \times 10^4$ cells ml^{-1}) in fresh medium and aliquots of this suspension plated onto glass coverslips. Cells were incubated overnight and then loaded with the Ca^{2+}-sensitive fluorescent dye Fura-2 by incubation (30–40 min) in medium containing the dye's membrane permeant, acetoxymethyl ester (AM) form (3 μM). A rise in $[Ca^{2+}]_i$ causes a corresponding rise in the Fura-2 fluorescence ratio recorded from Fura-2-loaded cells and this allows changes in $[Ca^{2+}]_i$ to be monitored using standard microspectrofluorimetric techniques (Grynkiewicz *et al*., 1985; Ko *et al*., 1994). In the present study, coverslips bearing Fura-2 loaded cells were mounted in a heated chamber mounted on the stage of a Nikon inverted microscope were they were superfused with HEPES-buffered saline. Fura-2 fluorescence ratios were recorded from single cells at 1.5 Hz. Increases in $[Ca^{2+}]_i$ were quantified by measuring the fluorescence ratio at the peak of a response and subtracting from it the ratio measured before stimulation.

Transepithelial anion secretion

Cells plated onto permeable supports fabricated from Millipore filters (2.2×10^5 cells cm^{-2}) were allowed to form polarized epithelial monolayers (4 days in culture) as described previously (Ko *et al*., 1996). Filters bearing such monolayers were mounted in Ussing chambers, bathed with bicarbonate-buffered saline and the current required to hold the transepithelial potential difference at 0 mV (short circuit current, I_{SC}) continuously measured and displayed. The effects of nucleotides were explored by adding these substances to the saline bathing the apical side of the epithelia. Previous work has shown that these compounds are essentially ineffective when added to the basolateral solution, and that nucleotide-evoked increases in I_{SC} reflect the secretion of anions into the apical solution (Ko *et al*., 1997). Such responses were quantified by measuring the current flowing at the peak of a response and subtracting the current measured before stimulation. In some experiments, epithelia were loaded with 1,2-bis-(2-aminophenoxy)-ethane-N,N,N′N′-tetraacetic acid (BAPTA) by adding the AM form (50 μM) of the compound to the solutions bathing the apical and basolateral sides of the epithelia and incubating cells in this solution for 45 min. This substance can abolish receptor mediated $[Ca^{2+}]_i$-transients by artificially increasing the $[Ca^{2+}]_i$-buffering capacity of the cytoplasm.

Simultaneous measurement of $[Ca^{2+}]_i$ and anion secretion

$[Ca^{2+}]_i$ and I_{SC} were measured simultaneously using a method similar to that described by Lazarowski *et al*. (1997). Aliquots (0.1 ml) of cell suspension containing 2×10^4 cells were seeded onto Transwell-Col filter membranes (pore diameter, 0.4 μm; Costar, USA) attached to O rings; the culture area was 0.1 cm^2. These assemblies were then floated on culture medium and cells were allowed to grow into confluent monlayers (3 days) that were loaded with Fura-2 by incubation (45 min, 37°C) in bicarbonate-buffered saline containing 3 μM Fura-2-AM and 1.6 μM pluronic F127. The epithelia took up very little Fura-2 under standard conditions and so inclusion of pluronic F127 was vital if Fura-2 fluorescence ratios were to be recorded. Membranes bearing Fura-2 loaded epithelia were then mounted in a miniature Ussing chamber the design of which allowed the apical and basolateral sides of the cell layers to be superfused independently. This chamber was mounted on the stage on a Nikon inverted microscope where the cells were viewed with a 40× extra long working distance objective. Fura-2 fluorescence ratios were recorded from a field containing 30–40 cells using a PTI Ratio-Master fluorescence system (Photon Technology International, NJ), whilst I_{SC} was simultaneously measured by a voltage-clamp amplifier (VCC 600; Physiologic Instruments, San Diego).

Data analysis

All data are presented as mean ± s.e. and the statistical significance of differences between mean values was tested by use of Student's *t* test. Values of *n* refer to the number of experiments in each group. The responses of experimental cells were always compared with the responses of age-matched, control cells at identical passage. EC_{50} values were estimated from sigmoid curves fitted to the experimental data using commercially-available software (Grafit 3.0, Erithacus Software Ltd., Staines, U.K.).

Results

Nucleotide-evoked $[Ca^{2+}]_i$-signals

Brief pulses (30 s, 1–300 μM) of UTP, ATP or 5-Br-UTP elicited concentration-dependent increases in $[Ca^{2+}]_i$ in single cells on coverslips. Whilst the responses elicited by maximally-effective concentrations of these nucleotides could not be distinguished from each other, the EC_{50} values were 2.4 ± 0.3 μM ($n = 5$), 2.3 ± 0.2 μM ($n = 6$) and 30.3 ± 5.9 μM ($n = 6$) respec-

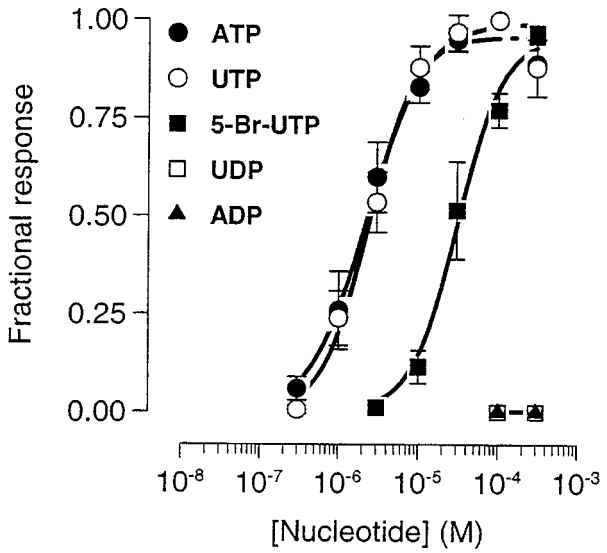

Figure 1 Pharmacological properties of nucleotide-evoked $[Ca^{2+}]_i$-signals in single cells. In each experiment, cells were stimulated with 30 s pulses of increasing concentrations of either ATP ($n = 6$), UTP ($n = 6$), 5-Br-UTP ($n = 6$), UDP ($n = 3$) or ADP ($n = 3$). These were delivered at intervals of at least 2 min and, at the end of experiments involving nucleotides other that UTP, the cells were also stimulated with a 30 s pulse of this nucleotide (100 μM) so that all increases in Fura-2 fluorescence ratio could be normalized to the response evoked by this standard stimulus. These data are plotted (mean with vertical lines showing s.e.) against the concentration of nucleotide used.

tively (Figure 1). Initial experiments ($n=5$) suggested that UDP may also increase $[Ca^{2+}]_i$ ($EC_{50}=62.0\pm23.9$ μM) but this response was abolished by pretreating the nucleotide with hexokinase/glucose (Figure 1). Similarly, earlier work had suggested that ADP may increase $[Ca^{2+}]_i$ (Ko *et al*., 1997), but this apparent response was also abolished if hexokinase/glucose treated ADP was used (Figure 1). The UDP- and ADP-evoked $[Ca^{2+}]_i$-signals thus appear to have been artefacts mediated by the UTP and ATP that contaminates commercially-available nucleotide diphosphates (see Nicholas *et al*., 1996; Harden *et al*., 1997). The UDP and ADP used in *all* subsequent experiments was, therefore, decontaminated using hexokinase/glucose (Nicholas *et al*., 1996).

Cross desensitization of ATP and UTP-evoked increases in $[Ca^{2+}]_i$

To explore the extent to which the ATP- and UTP-evoked $[Ca^{2+}]_i$ signals were subject to desensitization, cells were stimulated with two 30 s pulses of 100 μM ATP or 100 μM UTP separated by an interval of several minutes. Under control conditions (Figures 2a and 3b), each such stimulus evoked a transient rise in $[Ca^{2+}]_i$. Moreover, the response to the second stimulus was only slightly smaller than that evoked by the first, and so minimal spontaneous desensitization developed under these conditions. Further experiments, using age-matched cells at identical passage, showed that interposing a 5 min pulse of either 1 mM ATP or 1 mM UTP between the two test stimuli reduced the magnitude of the second response. The extent to which these manoeuvres reduced sensitivity was quantified by comparing the $[Ca^{2+}]_i$-signals evoked by the first and second test stimuli. This analysis showed that 1 mM ATP caused both autologous desensitization of the response to 100 μM ATP (Figure 2c, $72.6\pm5.2\%$, $n=5$, $P<0.001$) and cross-desensitization of the response to 100 μM UTP (Figure 2d, $67.5\pm7.9\%$, $n=7$, $P<0.01$). There was no significant difference in the magnitude of these effects. Moreover, 1 mM UTP also evoked identical loss of sensitivity to 100 μM ATP ($77.8\pm5.9\%$, $n=7$, $P<0.01$, Figure 2e) and 100 μM UTP ($87.7\pm3.4\%$, $n=5$, $P<0.01$, Figure 2f). These data are, therefore, consistent with the hypothesis that ATP and UTP increase $[Ca^{2+}]_i$ by acting upon a common receptor population (Ko *et al*., 1994).

Anion secretion evoked by UTP, 5-Br-UTP, UDP and ADP

Apical UTP, 5-Br-UTP, UDP and ADP increased I_{SC}. The response to each nucleotide consisted of a rapid rise to a

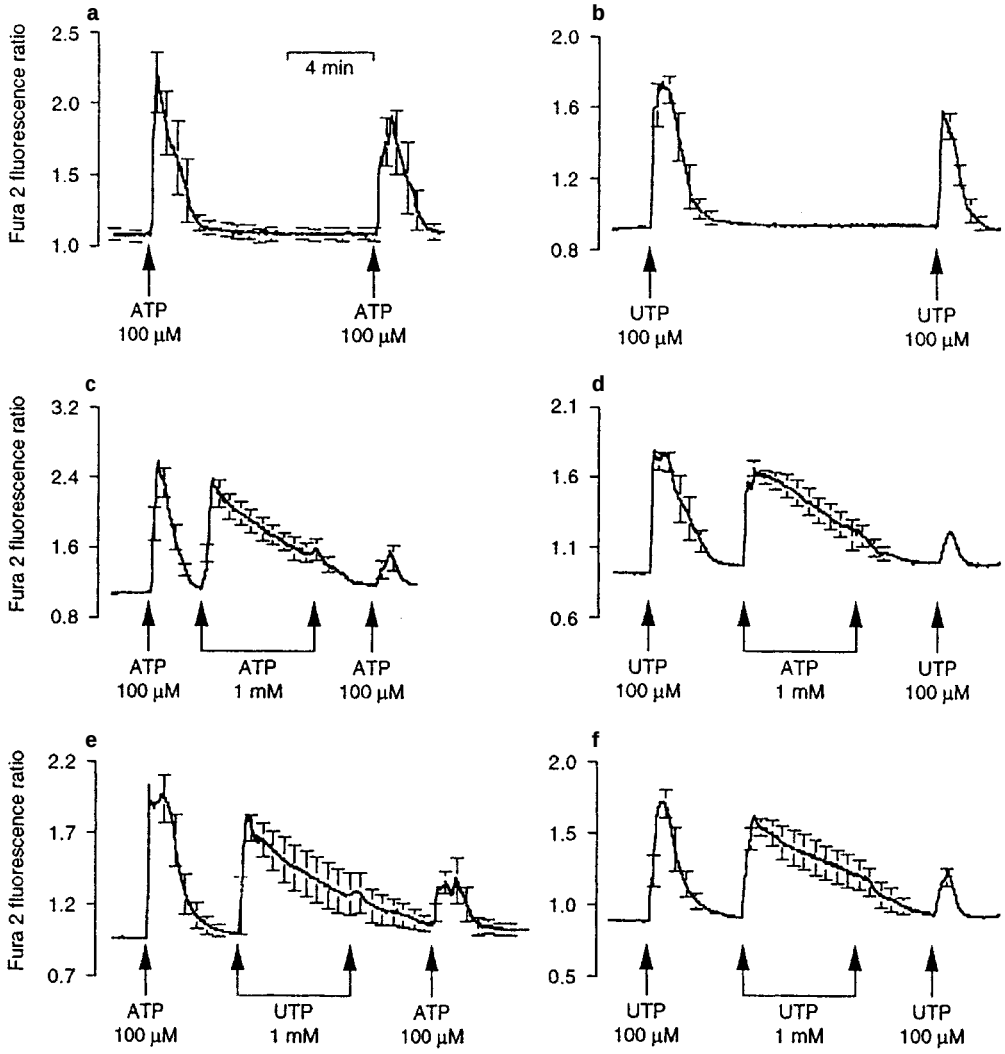

Figure 2 Cross-desensitization of UTP and ATP-evoked $[Ca^{2+}]_i$-signals. Each panel shows mean (vertical lines show s.e.) Fura-2 fluorescence ratios derived from at least 5 experiments in which single cells were stimulated with ATP and/or UTP as shown. The single arrows indicate the delivery of a 30 s pulse of the appropriate nucleotides.

clearly defined peak that was followed by an equally rapid fall that occurred despite the continued presence of agonist, and I_{SC} had, invariably, returned to the basal value after 5–10 min (see Ko *et al.*, 1997). The responses to each nucleotide were concentration-dependent (Figure 4) and the EC_{50} values for UTP, 5-Br-UTP, UDP and ADP were calculated to be 2.3 ± 0.2 μM, 2.7 ± 1.3 μM, 1.1 ± 0.2 μM and 82.2 ± 6.5 μM, respectively (Figure 3). Earlier work (Ko *et al.*, 1997) had shown that pyrimidine nucleotides could increase I_{SC}. by activating receptors that did not desensitize during exposure to ATP. To establish the pharmacological properties of this receptor population, epithelia were first stimulated with 100 μM ATP, which increased I_{SC}. by 62.2 ± 1.6 μA cm^{-2} ($n=105$), and then exposed to nucleotides other than ATP once the current had returned to its basal value (5–10 min). ATP-epithelia prestimulated continued to respond to UTP, 5-Br-UTP, UDP and ADP although the maximal responses were smaller than normal (Figure 3) and, under these conditions, the EC_{50} values for UTP, 5-Br-UTP and UDP were 10.9 ± 0.1 μM, 4.1 ± 2.7 μM and 2.8 ± 0.8 μM, respectively. Although ADP evoked a clear response in the ATP-prestimulated cells, it was not possible to estimate EC_{50} accurately as even the highest concentration tested did not evoke a maximal response (Figure 3d).

The ATP-evoked rise in I_{SC}

ATP evoked a concentration-dependent increase in I_{SC} ($EC_{50}=11.0\pm0.6$ μM) that was subject to essentially complete (>95%) autologous desensitisation (Figure 4). Moreover, the response to 100 μM ATP seen in UTP-prestimulated (100 μM) epithelia was also smaller than normal (control: 52.2 ± 2.9 μA cm^{-2}, prestimulated: 3.6 ± 0.4 μA cm^{-2}) confirming (Ko *et al.*, 1997) that UTP also causes loss of sensitivity (93.1 ± 0.7%) to ATP (Figure 5a). Indeed, UTP desensitized the cells as effectively as ATP itself. Sensitivity to ATP was also reduced by prestimulating the epithelia with 100 μM 5-Br-UTP (control:

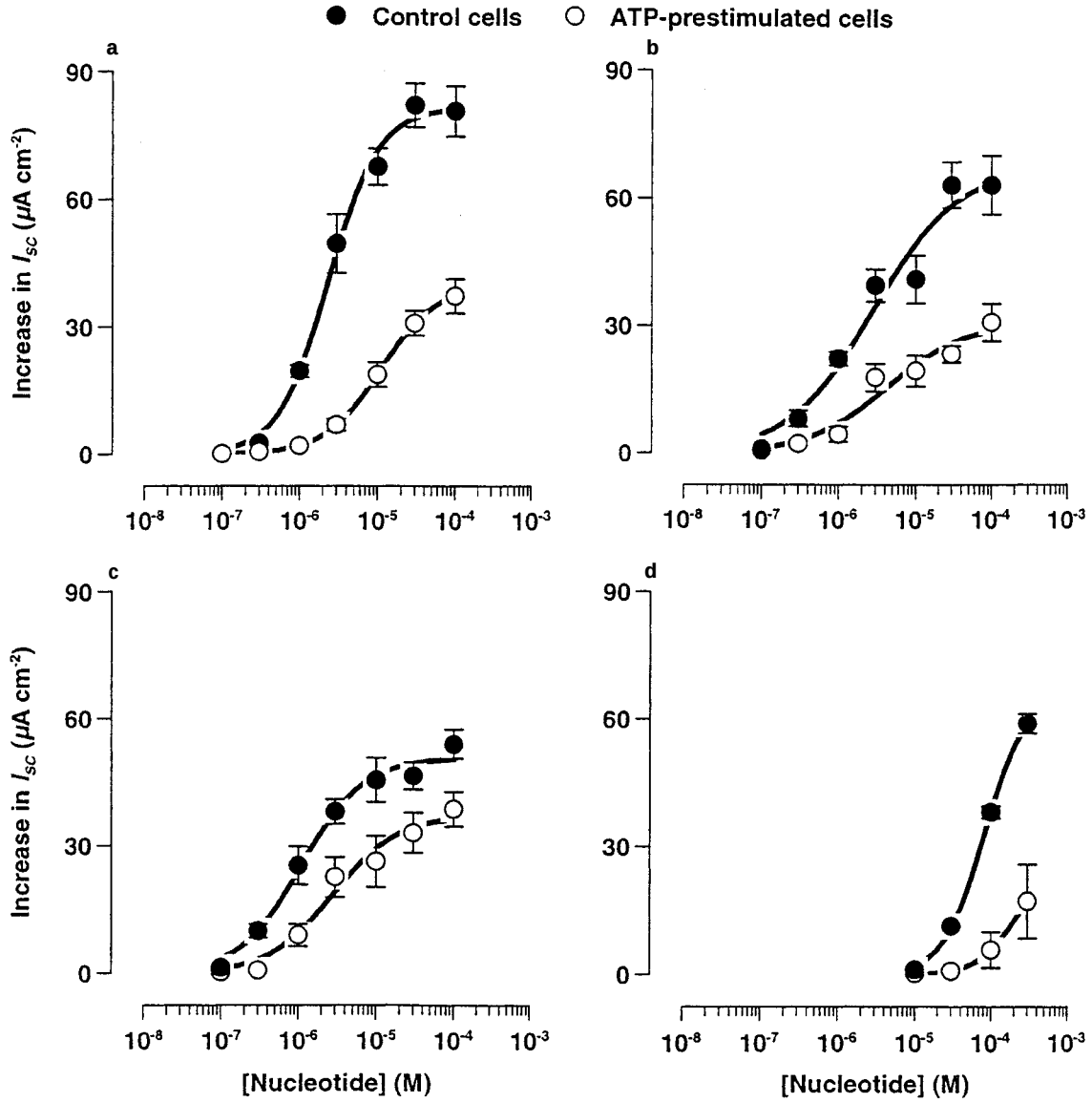

Figure 3 Epithelial monolayers on Millipore filters were stimulated by adding UTP (a), 5-Br-UTP (b), UDP (c) or ADP (d) to the apical solution and the resultant increases in I_{SC} quantified and plotted against the concentration of nucleotide used. These experiments were undertaken using control epithelia and age-matched epithelia, at identical passage, that had been exposed to ATP (100 μM) 5–10 min before stimulation with the test nucleotides. Each data point is mean and vertical lines s.e. of 4–8 independent observations.

47.4±4.7 μA cm^{-2}, prestimulated: 8.5±1.7 μA cm^{-2}) or 1 mM ADP (control: 51.7±5.5 μA cm^{-2}, prestimulated: 8.9±3.1 μA cm^{-2}) although these nucleotides were less effective desensitizing agonists (85.1±3.6% and 82.8±3.1%, respectively, $P<0.05$ for both) than UTP. Moreover, 100 μM UDP also elicited loss of sensitivity to ATP (Figure 5b, control: 65.1±6.9 μA cm^{-2}, UDP-prestimulated: 26.8±2.9 μA cm^{-2}) but this substance was even less effective (58.9±4.4%) than 5-Br-UTP ($P<0.05$) or ADP ($P<0.05$). As earlier work had indicated that the effects of UDP were mediated, primarily, *via* an ATP-insensitive receptor (Ko *et al*., 1997), we undertook a more complete analysis of this interaction by quantifying the responses to ATP (1–100 μM) in UDP-prestimulated cells. These experiments (Figure 4) confirmed that UDP evoked desensitization and showed that the EC_{50} for ATP in UDP-prestimulated cells was 30.6±1.1 μM. Both ADP and UDP thus cause some loss of sensitivity to ATP.

Cross-desensitization between UDP and ADP

To explore the possibility that ADP and UDP increase I_{SC} by acting upon a common receptor, cultured epithelia were first stimulated with 100 μM UDP. This increased I_{SC} by 74.3±3.0 μA cm^{-2} ($n=5$) and, although subsequent addition of 100 μM ADP evokes a clear response (Figure 5c), the response (7.2±1.2 μA cm^{-2}) was smaller ($P<0.001$) than control (36.3±2.0 μA cm^{-2}). UDP thus caused the cells to lose sensitivity to ADP. The extent to which ADP could desensitize the epithelia to UDP was explored ($n=5$) by administering the nucleotides in the reverse sequence (Figure 5d). In these experiments, a maximally-effective concentration of ADP (1 mM) increased I_{SC} by 60.4±6.0 μA cm^{-2} and, although the cells subsequently responded to 100 μM UDP (22.56±3.25 μA cm^{-2}), the response was smaller ($P<0.01$) than control (50.1±2.5 μA cm^{-2}, $n=5$). Analysis of these data showed that UDP evoked loss of sensitivity to ADP more effectively (80.1±3.3%) than ADP desensitized the cells to UDP (45.0±6.5%), suggesting that the nucleotides may not act upon identical receptor populations.

Cross-desensitization between ATP and bradykinin

Bradykinin (10 μM) evoked a transient rise in I_{SC} (26.7 μA cm^{-2}, $n=5$) that could not be distinguished from the responses evoked by nucleotides. Although the bradykinin-stimulated epithelia responded to 100 μM ATP (48.7±1.9 μA cm^{-2}), the response was smaller than that observed in age-matched control epithelia (75±6.7 μA cm^{-2}, $P<0.05$). Bradykinin thus evokes ~35 % desensitization of the response to ATP.

Effects of BAPTA upon the responses to ATP and UDP

BAPTA-loaded epithelia continued to respond to ATP but the increase in I_{SC} (17.5±3.3 μA cm^{-2}, $n=6$) was smaller ($P<0.001$) than control (65.1±6.9 μA cm^{-2}, $n=6$). However, separate, using Fura-2-loaded cells on coverslips, experiments ($n=4$) showed that the calcium signals evoked by 100 μM ATP were completely abolished in BAPTA-loaded cells. BAPTA also inhibited UDP-evoked increase in I_{SC} (control: 42.3±5.5 μA cm^{-2}, $n=5$, BAPTA-loaded: 3.1±0.9 μA cm^{-2}, $n=6$, $P<0.0001$) and analysis of these data showed BAPTA inhibited this response (92.8±2.1%) more effectively ($P<0.05$) than the response to ATP (73.1±5.1%).

Simultaneous measurement of I_{SC} *and* $[Ca^{2+}]_i$

Experiments ($n=6$) in which $[Ca^{2+}]_i$ and I_{SC} were measured simultaneously confirmed that UTP (100 μM) increased I_{SC} and, as anticipated, this response was accompanied by increased $[Ca^{2+}]_i$ (Figure 6). This work also confirmed that UDP (100 μM) increased I_{SC} (Figure 6). However, whilst this nucleotide failed to increase $[Ca^{2+}]_i$ in cells on coverslips (Figure 1), UDP-evoked anion secretion in polarized cells was consistently accompanied by a clear rise in $[Ca^{2+}]_i$ (Figure 6). Directly analogous experiments ($n=6$) showed that the ADP-evoked (1 mM) increase in I_{SC} was also accompanied by increased $[Ca^{2+}]_i$.

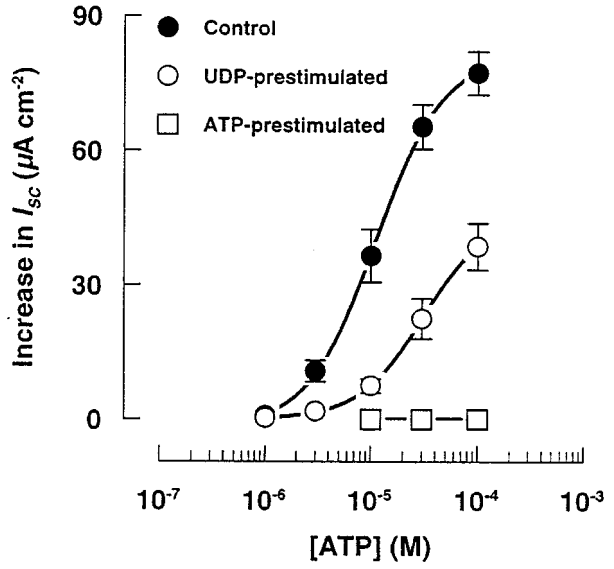

Figure 4 ATP-evoked (1–100 μM) increases in I_{SC} were quantified and plotted against the concentration of nucleotide used. Experiments were undertaken with control epithelia and epithelia that had been prestimulated with 100 μM ATP or 100 μM UDP. Each point is mean and vertical lines s.e. of at least 6 independent observations.

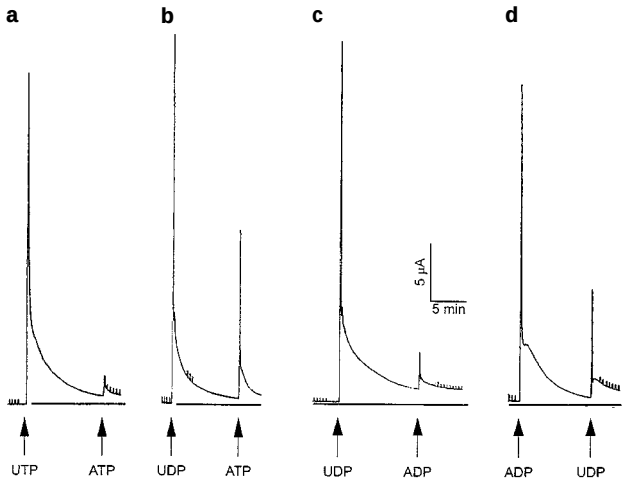

Figure 5 Cross-desensitization between different nucleotides. Each panel shows a record of I_{SC} made whilst the appropriate nucleotides (all 100 μM) were delivered to the apical side of the cell layers as indicated. In each instance, essentially identical responses were obtained in at least 5 independent experiments.

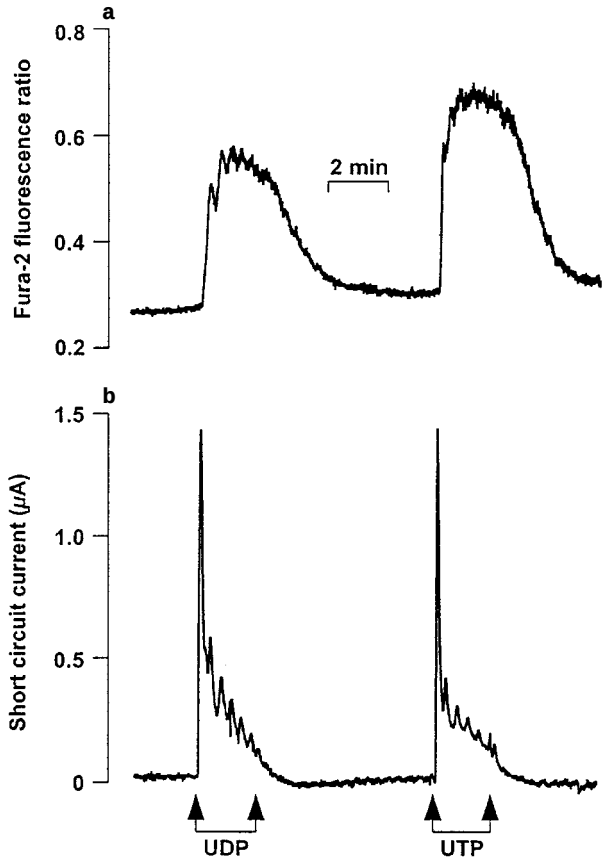

Figure 6 (a) A continuous record of Fura-2 fluorescence ratio made from a confluent epithelial monolayer, whilst (b) shows a record of I_{SC} made concurrently from the same culture. UDP and UTP (both 100 μM) were added to the apical solution as indicated. Essentially identical responses were seen in 4 independent experiments.

Discussion

The present study confirmed that extracellular nucleotides elicit $[Ca^{2+}]_i$-signals in single E/92/3 epithelial cells on coverslips and that apical nucleotides also increase I_{SC} in polarized cultures of these cells (Ko *et al*., 1994; 1997; Wilson *et al*., 1996). However, whilst the $[Ca^{2+}]_i$-signals were mediated by a single receptor population, the increases in I_{SC} clearly involved more than one receptor subtype. Cross desensitization experiments indicated that $P2Y_2$ receptors were present in both preparations, but that polarized cells expressed an additional receptor subtype that was insensitive to ATP but which could be activated by certain other nucleotides with a rank order of potency of UDP > 5-Br-UTP > UTP > ADP. Apical receptors with properties very similar to these have recently been identified in human airway epithelia and cultured colonocytes. So there is now a body of evidence to suggest that polarized epithelia may express an unidentified, apical P2Y receptor subtype that is insensitive to ATP but potently activated by UDP (Ko *et al*., 1997; Lazarowski *et al*., 1997; Inoue *et al*., 1997).

It is interesting, in this context, that two previously undescribed P2Y receptor subtypes, $P2Y_4$ receptors and $P2Y_6$ receptors, have recently been cloned and characterized. Both are essentially insensitive to ATP but the $P2Y_6$ receptor is clearly activated by UDP but has some sensitivity to ADP (Chang *et al*., 1995; Communi *et al*., 1995; 1996; Nguyen *et al*., 1995; Nicholas *et al*., 1996; Communi & Boeymans, 1997). Although it is therefore very tempting to assume that it is these receptors that are present in the apical membranes of polarized epithelia, not all of our data were consistent with this hypothesis. ATP-prestimulated epithelia thus continued to respond to UTP (see also Ko *et al*., 1997), whilst $P2Y_6$ receptors are essentially insensitive to nucleotide triphosphates (Communi *et al*., 1996; Nicholas *et al*., 1996; Communi & Boeymans, 1997). Moreover, cross-desensitization experiments indicated that UDP and ADP may evoke loss of sensitivity to ATP and raised the possibility that these nucleotide diphosphates may not act upon the same receptor populations. However, the pharmacological properties of P2Y receptors are notoriously difficult to discern from cross-desensitization experiments, as the different subtypes invariably display overlapping patterns of agonist selectivity (Burnstock, 1990; Dubyak & El-Moatassim, 1993). Furthermore, we cannot exclude the possibility that at least some of the desensitizing actions which we now report may have been due to intracellular events, such as the depletion of intracellular calcium stores. Indeed, our observation that bradykinin, a non-nucleotide agonist, induce loss of sensitivity to ATP, suggests strongly that such interactions do occur. We are, therefore, currently undertaking molecular studies of these cells which aim to establish a formal identification of the P2Y receptor subtypes present.

All P2Y receptors allow nucleotides to increase $[Ca^{2+}]_i$ (Dubyak & El-Moatassim, 1993; Nicholas *et al*., 1996) and, in epithelia, it is generally assumed to be this which allows nucleotides to evoke secretion (e.g. Petersen, 1992). The present data therefore presented an apparent contradiction: how could UDP and ADP evoke secretion if they are unable to raise $[Ca^{2+}]_i$? In an attempt to resolve to this paradox, we explored the possibility that the nucleotides may act *via* a $[Ca^{2+}]_i$-independent mechanism. There are precedents for this because, as well as raising $[Ca^{2+}]_i$, the apical P2Y receptors expressed by other epithelia seem to allow nucleotides to activate anion channels *via* a mechanism which does not involve a cytoplasmic second messenger (Stutts *et al*., 1994; Guo *et al*., 1995; 1997). Our studies of BAPTA-loaded epithelia certainly showed that the response to ATP consisted of $[Ca^{2+}]_i$-dependent and $[Ca^{2+}]_i$-independent components. However, the response to UDP appeared to be entirely due to increased $[Ca^{2+}]_i$. This clear result raised the possibility that the polarized cells may express $[Ca^{2+}]_i$-mobilising receptors that are not present in the single cells and this hypothesis was verified by a final series of technically difficult experiments in which I_{SC} and $[Ca^{2+}]_i$ were measured simultaneously.

Receptors that allow UDP and ADP to evoke anion secretion thus appear to be expressed only by cells that have adopted a polarized phenotype, whilst the well documented $P2Y_2$ receptor seems to be present in both preparations (Ko *et al*., 1994; Wilson *et al*., 1995). It is therefore possible that many epithelia cell types may express such $[Ca^{2+}]_i$-mobilizing, UDP-sensitive receptors if maintained under the appropriate conditions (see Inoue *et al*., 1997; Lazarowski *et al*., 1997). These receptors may, therefore, provide a component to the apical signalling system the importance of which has been grossly underestimated by the many previous studies of cells cultured under standard conditions (Dubyak & El-Moatassim, 1993).

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