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Purine and pyrimidine nucleotide receptors in the apical membranes of equine cultured epithelia

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1 The short circuit current (I_{SC}) technique was used to quantify electrolyte transport by equine cultured sweat gland epithelia. Adenosine 5′-triphosphate (ATP) and certain related compounds, caused transient increases in I_{SC} when added to the apical solution. The order of potency was uridine triphosphate (UTP) > ATP > ADP > > AMP = adenosine.

2 The responses to apical nucleotides were due to chloride and bicarbonate secretion and were reduced in pertussis toxin-treated cells. P_2-receptors sensitive to uridine 5′-triphosphate (UTP), that interact with inhibitory G proteins, therefore appear to be present in the apical membrane.

3 Responses to ATP and UTP were reduced in cells loaded with BAPTA, a calcium chelator. BAPTA attenuated the response to ATP more than the response to UTP suggesting that these nucleotides may not act via a common pathway.

4 Cross-desensitization experiments indicated that two populations of UTP-sensitive receptor were present. One was sensitive to UTP and ATP, whereas the second was sensitive only to UTP. Uridine diphosphate appeared to activate the ATP-insensitive receptor population selectively.

5 These data suggest that apical pyrimidinoceptors may be expressed by these cells. The physiological role of these receptors is unknown but they may allow the autocrine regulation of epithelial function.

Keywords: UTP receptors; nucleotide receptors; pertussis toxin; Ussing chambers; cell culture; chloride secretion; stimulus-secretion coupling; sweat glands

Introduction

Extracellular adenosine 5′-triphosphate (ATP) increases intracellular free calcium ($[Ca^{2+}]_i$) in a cell line (E/92/3, Wilson *et al*., 1993) derived from the secretory epithelium of the equine sweat gland and this response appears to be mediated via purine receptors belonging to the P_{2U} subclass (Ko *et al*., 1994). Whilst the physiological significance of these receptors is not obvious, the increase in $[Ca^{2+}]_i$ allows ATP to regulate the ionic permeability of the plasma membrane (Wilson *et al*., 1995). We therefore proposed that epithelial P_{2U} receptors may allow ATP released from periglandular nerves to regulate transepithelial salt and water transport (Ko *et al*., 1994; Wilson *et al*., 1995). Further work showed that E/92/3 cells consistently formed polarized monolayers when grown on permeable supports and that the transport of electrolytes across such cultured epithelia could be quantified by standard, electrometric techniques (Ko *et al*., 1996). In the present study, we have therefore used this preparation to establish the extent to which nucleotides are able to regulate ion transport in these cells. Some of the data have been presented to be Physiological Society (Ko *et al*., 1995).

Methods

Quantification of transepithelial electrolyte transport

Standard techniques were used to maintain E/92/3 cells in serial culture. For experiments, cells were removed from culture flasks by trypsin/EDTA and resuspended in fresh culture medium. Aliquots of this cell suspension were seeded into the wells formed by sticking Sylgard rings onto Millipore filters (Wong, 1988) and these assemblies were then carefully floated on culture medium in Petri dishes, where the cells were allowed to grow (37°C, 5% CO_2 in air) for 4 days. Cells were plated at a density of 2.2×10^5 cells cm^{-2} as previously described, ultrastructural studies had shown that polarized monolayers consistently formed under these conditions, and that the upward facing aspect of these cultured epithelia was equivalent to the apical membrane of the parent epithelium (Ko *et al*., 1996). Pertussis toxin-treated epithelia were prepared by pipetting toxin-containing medium (100 ng ml^{-1}) into the well after 3 days in culture. An equal volume of standard medium was pipetted onto control epithelia to ensure that simply disturbing the cells did not affect their sensitivity. All monolayers were then cultured for a further 18–24 h. Preliminary experiments had shown that simply adding this toxin to the medium supporting the filters had no effect.

Filters bearing cultured epithelia were mounted in Ussing chambers; bathed with physiological salt solution (37°C) and the current required to hold transepithelial potential difference at 0 mV (short circuit current, I_{SC}) continuously displayed on a pen recorder (Wong, 1988). Transepithelial resistance was monitored by measuring currents evoked by transient excursions (0.1 mV) from this holding potential. The use of Ussing chambers requires that the apical and basolateral sides of the cell layer are bathed by separate solutions. In the present study, the apical and basolateral baths each contained 20 ml of saline which remained in contact with the cells throughout each experiment. Both reservoirs were normally bubbled with 95% O_2/5% CO_2 to maintain pH and ensure constant agitation. Epithelia were stimulated by adding drugs into the appropriate bath. In some experiments, epithelia were loaded with the calcium chelator 1,2-bis-(2-aminophenoxy)-ethane-N,N,N′N′-tetraacetic acid (BAPTA) by adding the compound's membrane permeant, acetoxymethyl ester form, (50 μM) to the apical and basolateral solutions. The epithelia were incubated for 45 min before agonists were added to the appropriate bath. The effects of barium were explored by exposing the basolateral side of the epithelium to $BaCl_2$ for 5 min, whereas amiloride was added to the apical solution

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5 min before the epithelia were stimulated. TPA and staurosporine were added to the apical and basolateral solutions 10 min before the addition of agonists.

Solutions and chemicals

The culture medium was William's medium E containing L-glutamine (1 mM), penicillin (100 i u ml^{-1}), streptomycin (100 μg ml^{-1}), bovine insulin (5 μg ml^{-1}), epidermal growth factor (0.1 μg ml^{-1}), hydrocortisone (10 ng ml^{-1}), transferrin (5 μg ml^{-1}) and sodium selenite (5 ng ml^{-1}). Medium containing pertussis toxin (Sigma) was prepared by adding the toxin to otherwise complete medium immediately before use. The standard, 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 11.1. Its pH was 7.3–7.4 when bubbled with 5% CO_2/95% O_2. In order to prevent the precipitation of $BaSO_4$ and $BaPO_4$, the saline used in experiments involving Ba^{2+} contained no KH_2PO_4, and $MgSO_4$ was replaced by $MgCl_2$. Chloride-free saline was prepared by iso-osmotically replacing chloride with gluconate, and $NaHCO_3$ was also replaced by 4-(2-hydroxyethyl)-1-piperazineethanesulphononic acid (HEPES) in the chloride and bicarbonate-free solution. This was adjusted to 7.4 with NaOH and was bubbled with 100% O_2. The bicarbonate-buffered, sodium-free solution was prepared by iso-osmotically replacing sodium with N-methyl-D-glucammonium. Stock solutions of ATP, uridine 5′-triphosphate (UTP), uridine 5′-diphosphate (UDP), ADP, adenosine 5′-monophosphate (AMP) and adenosine (all 100 mM) were prepared in distilled water, whereas amiloride (100 mM), 12-*O*-tetradecanoyl phorbol 13-acetate (TPA, 10 mM) and staurosporine (2 mM) were dissolved in dimethylsulphoxide. These solutions were stored at −20°C. Cell culture reagents were purchased from Gibco Laboratories (New York, U.S.A.) whereas nucleotides, drugs and general laboratory chemicals were from Sigma Chemical Co. (St. Louis, U.S.A.).

Data analysis

Positive currents were defined as those carried by anions moving from the basolateral to the apical compartments and are shown as upward deflections of the traces. Increases in I_{SC} were quantified by subtracting the current measured immediately before the addition of a drug from the current measured at the peak of a response. Pooled data (means ± s.e.) are expressed as increases in current per square centimetre of epithelium (μA cm^{-2}). The epithelial area exposed was 0.45 cm^2. Values of n refer to the number of epithelial monolayers in each group. Experiments were designed so that the responses of experimental cells were always compared with the responses of age-matched control epithelia that had been exposed to the appropriate, solvent vehicle. Unless otherwise stated, each monolayer was stimulated only once and the significance of differences between mean values are tested by Student's unpaired t test. EC_{50} values were estimated from sigmoid curves fitted to the experimental data with commercially-available software (Grafit 3.0, Erithacus Software Ltd., Staines, U.K.).

Results

Transepithelial resistance ranged from 113–900 Ωcm^2 ($n>200$) and, under open circuit conditions, the epithelia generated a spontaneous potential difference of 0.12 ± 0.02 mV (apical side negative). At the onset of the experiments, basal I_{SC} was 0.13 ± 0.04 μA cm^{-2}. Initial experiments showed that ATP (1–300 μM) increased I_{SC} when added to the solution bathing either side of the epithelium but that much larger responses were evoked by apical ATP (Figure 1). These responses are described separately.

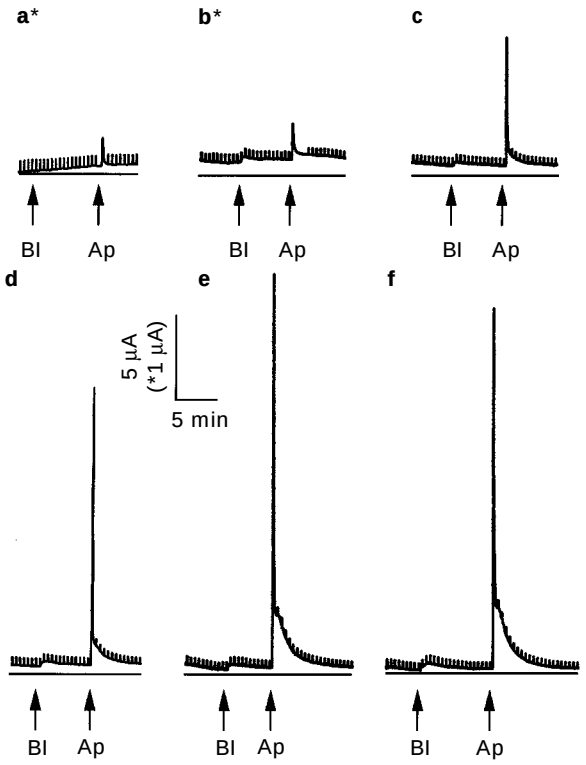

Figure 1 Effects of ATP upon I_{SC}. Each panel shows a continuous record of I_{SC} made from a separate epithelial monolayer in which ATP was first added to the solution bathing the basolateral aspect of the cell layer (Bl) and then to the solution bathing the apical side (Ap). The final concentrations of ATP were 1 μM (a), 3 μM (b), 10 μM (c), 30 μM (d), 100 μM (e) and 300 μM (f). The horizontal line in each record indicates zero current. Note that the records presented in (a) and (b) were made at higher gain.

Properties of the basolateral response

The basolaterally-mediated response usually had a slow onset although there was variability in its form and magnitude, and rapid responses were seen in some instances (see e.g. Wilson *et al*., 1996). The response to basolateral ATP (1 mM) was reduced during exposure to chloride-free saline (control: 8.5 ± 2.3 μA cm^{-2}, $n=6$; chloride-free: 1.4 ± 0.2 μA cm^{-2}; $n=6$; $P<0.001$) and the response that persisted under these conditions was essentially abolished when external bicarbonate was also absent (<1 μA cm^{-2}, $n=5$, $P<0.05$). The cells also responded to basolateral UTP and this nucleotide appeared to be as potent as ATP although reliable estimates of EC_{50} could not be made as the responses were small and variable (Figure 2). The cells were essentially unresponsive to basolateral ADP (Figure 2), AMP (300 μM) and adenosine (300 μM). BAPTA did not affect the responses to basolateral ATP (control: 1.5 ± 0.4 μA cm^{-2}, $n=22$; BAPTA-treated: 1.2 ± 0.2 μA cm^{-2}, $n=15$) or UTP (control: 2.6 ± 0.3 μA cm^{-2}, $n=17$; BAPTA-treated: 2.5 ± 0.3 μA cm^{-2}, $n=11$).

Properties of the apical response

Apical ATP evoked a rise in I_{SC} that occurred with no discernible latency and reached a clearly defined peak after ca 10 s. However, once this peak was reached I_{SC} fell until, after 5 min, it had returned to basal levels despite the continued presence of ATP (Figure 1). The magnitude of this response was concentration-dependent (Figure 1) and EC_{50} was estimated to be 42.0 ± 3.3 μM (Figure 3). The epithelia also responded to the pyrimidine nucleotide UTP, although analysis of the data showed the cells to be ca 13 times more sensitive to this nucleotide than to ATP (EC_{50} = 3.2 ± 0.7 μM, Figure 3). However, the responses to maximally effective concentrations

of UTP and ATP could not be distinguished. The cells also responded to ADP, but it was not possible to estimate EC_{50} for this compound, as the highest concentration tested did not evoke a maximal response (Figure 3). High concentrations of AMP or adenosine evoked only small responses (Figure 3). The rank order of potency amongst the tested compounds was therefore UTP > ATP > ADP > AMP = adenosine.

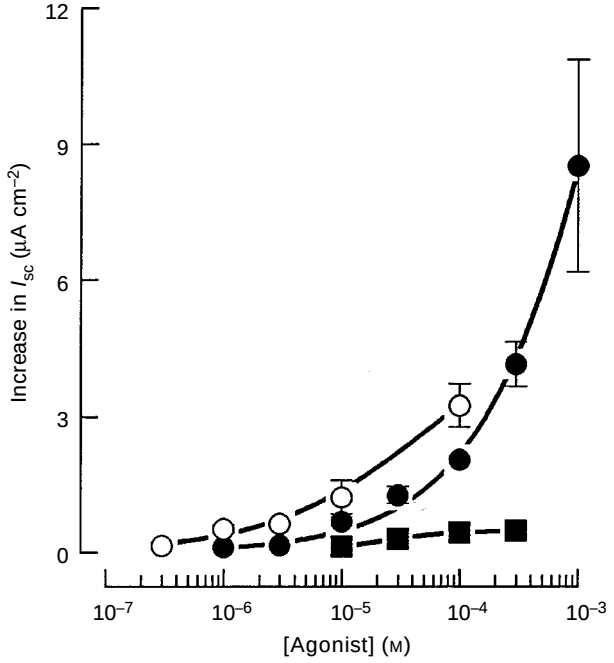

Figure 2 Data from experiments in which ATP (●), UTP (○) or ADP (■) were added to the solution bathing the basolateral side of the monolayers. Each point is the mean of between 4 and 11 observations; vertical lines show s.e.

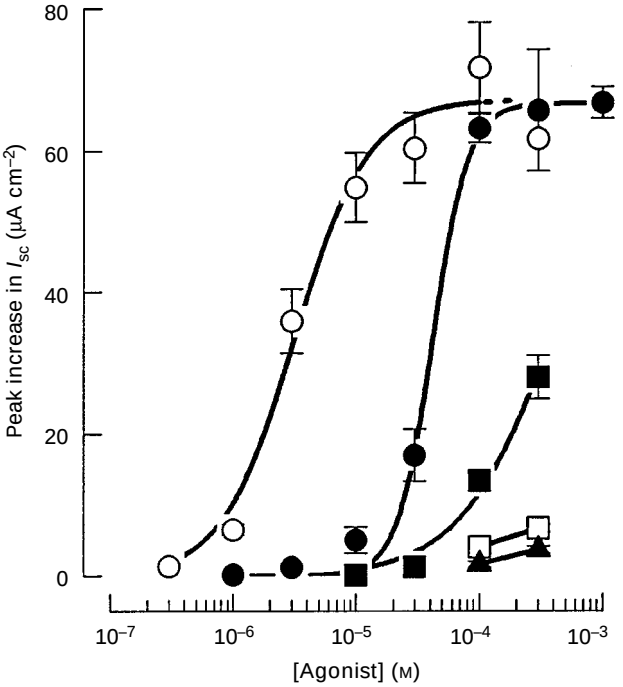

Figure 3 Effects of ATP and related compounds when added to the apical solution. Peak increases in I_{SC} evoked by ATP (●), UTP (○), ADP (■), AMP (□) or adenosine (▲) were plotted against the concentration of agonist used. Each point is the mean derived from between 4 and 12 observations; vertical lines show s.e.

The response to 1 mM ATP was unchanged when the apical solution was replaced with Na^+-free saline (control: 56.1 ± 4.1 μA cm^{-2} $n=6$, Na^+-free: 75.2 ± 8.1 μA cm^{-2}, $n=6$) and responses to ATP were also unaffected by apical addition of 0.1 mM amiloride, an inhibitor of epithelial sodium transport (control: 60.7 ± 4.8 μA cm^{-2}, $n=8$; amiloride: 63.5 ± 6.1 μA cm^{-2}, $n=8$). The positive currents evoked by ATP cannot, therefore, be attributed to electrogenic transport of sodium from the apical to the basolateral solutions. However, further experiments showed that the responses to 100 μM ATP were attenuated when epithelia were bathed with bicarbonate-buffered, chloride-free solution (control: 66.7 ± 2.9 μA cm^{-2}, $n=5$, Cl^--free: 14.7 ± 3.0 μA cm^{-2}, $n=5$, $P<0.0001$). The small response that persisted under these conditions was further reduced to 1.9 ± 0.5 μA cm^{-2} ($n=5$, $P<0.005$) when bicarbonate was also absent.

Epithelia treated with 5 mM Ba^{2+} continued to respond to ATP although the response was reduced by ca 12% (control: 60.1 ± 2.03 μA cm^{-2}, $n=25$, Ba^{2+}-treated: 52.31 ± 7.64 μA cm^{-2}, $n=9$, $P<0.05$). Pertussis toxin-treated epithelia also responded to UTP but the maximal response seen in these cells was only ca 35% of the control value (Figure 4). Transepithelial resistance slowly fell from 705 ± 71 Ωcm^2 to $222 \pm 26.\Omega$ cm^2 ($P<0.02$, paired t test) when cell layers were exposed to 0.16 μM TPA. Although these cells continued to respond to 100 μM UTP (Figure 5), the response (21.0 ± 1.2 μA cm^{-2}, $n=4$) was smaller ($P<0.0001$) than normal (67.2 ± 4.7 μA cm^{-2}, $n=4$). Responses to 100 μM UTP were unaffected (68.6 ± 2.8 μA cm^{-2}, $n=4$) by 0.1 μM staurosporine (Figure 5). Monolayers loaded with the calcium chelator BAPTA continued to respond to 100 μM ATP (control: 62.1 ± 2.1 μA cm^{-2}, $n=22$, BAPTA-loaded, 12.5 ± 1.2 μA cm^{-2}, $n=13$) or 100 μM UTP (control: 73.8 ± 2.2 μA cm^{-2}, $n=11$, BAPTA-loaded, 25.7 ± 2.7, $n=11$) although the responses to both agonists were always smaller than normal in the BAPTA-treated epithelia. However, analysis of these data showed that BAPTA reduced the response

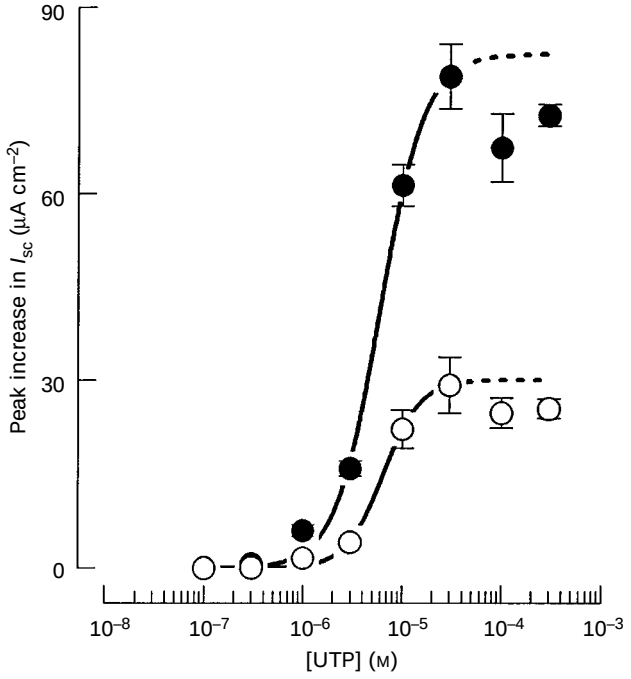

Figure 4 Effects of pertussis toxin upon the UTP-evoked increase in I_{SC}. Responses to UTP were quantified and plotted against the concentration of UTP used. Data from both control (●) and pertussis toxin-treated (○) cells at identical passage are presented as mean where each point is the mean of between 4 and 8 observations; vertical lines show s.e. All data points obtained derived from pertussis toxin-treated cells differed significantly ($P<0.05$) from the appropriate data points derived from control epithelia.

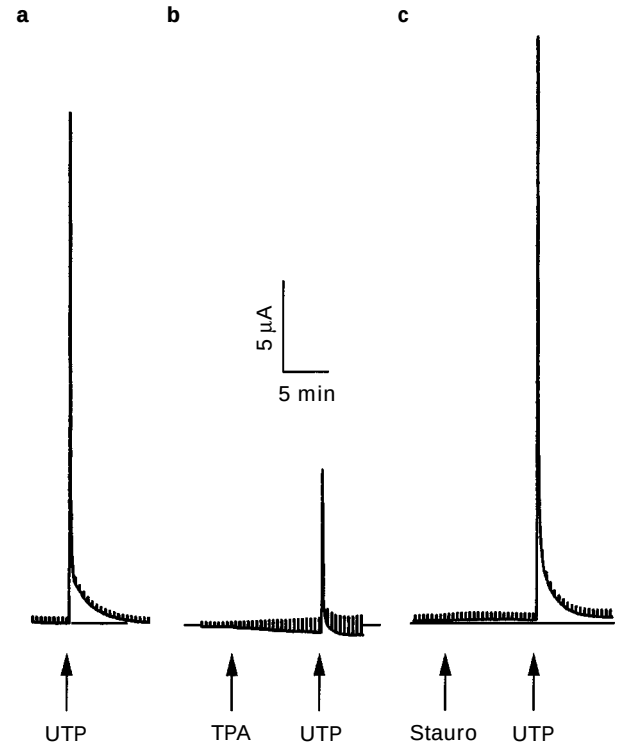

Figure 5 Effects of substances that modify the activity of protein kinase C (PKC) upon responses to UTP. (a) A typical response to UTP in a control culture, essentially identical records were obtained in 4 experiments. Responses to UTP in cultures that had been preincubated (10 min) in saline containing either 0.16 μM TPA ($n=4$) or 0.1 μM staurosporine (Stauro, $n=4$) are presented in (b) and (c), respectively. Horizontal lines indicate zero current.

to ATP ($80\pm2\%$ inhibition, $P<0.001$) more effectively ($P<0.02$) than the response to UTP ($65\pm4\%$ inhibition, $P<0.001$).

Cross-desensitization experiments

Epithelia were first stimulated by adding 100 μM ATP to the apical bath, and a second aliquot of this nucleotide (100 μM) was added to the apical solution once the I_{SC} had returned to basal levels. Although both stimuli evoked increases in I_{SC} (Figure 6a), the initial response (87.4 ± 8.5 μA cm^{-2}) was much larger ($P<0.002$, paired t test, $n=5$) than the second (3.8 ± 0.1 μA cm^{-2}). The record presented in Figure 6b shows that repeatedly adding 100 μM UTP to apical bath evoked a very similar pattern of response (first response: 73.3 ± 2.1 μA cm^{-2}, second response: 2.3 ± 0.5 μA cm^{-2}, $P<0.02$, paired t test, $n=4$). Subsequent experiments explored the extent to which ATP and UTP caused cross-desensitization. In the first of such experiments (Figure 6c) apical UTP (100 μM) increased I_{SC} (71.5 ± 0.5 μA cm^{-2}, $n=5$) but the response seen when these UTP-stimulated cells were subsequently exposed to 100 μM ATP was too small to be quantified (<1 μA cm^{-2}). However, experiments in which these nucleotides were added in the reverse sequence, showed clearly that ATP-stimulated epithelia (100 μM, increase in $I_{SC}=119.7\pm12.8$ μA cm^{-2}) consistently ($n=5$) responded to 100 μM UTP (99.0 ± 8.9 μA cm^{-2}). However, the response to UTP seen under these conditions was smaller than normal ($P<0.001$). Experiments in which this protocol was repeated with pertussis toxin-treated cells ($n=4$) showed that this toxin inhibited the initial response to ATP ($56.3\pm3.2\%$, $P<0.005$) as effectively as the subsequent response to UTP ($55.5\pm5.4\%$, $P<0.005$).

The epithelia also responded to apical UDP. Experiments ($n=3$) in which 100 μM concentrations of UDP were repeatedly added to the apical bath showed that this nucleotide, in common with ATP and UTP, caused homologous desensitization (first response: 34.0 ± 6.4 μA cm^{-2}, second response: <1 μA cm^{-2}, $P<0.05$, paired t test). Epithelia that had been stimulated with 100 μM UTP (71.6 ± 10.2 μA cm^{-2}) became essentially unresponsive to 100 μM UDP (<1 μA cm^{-2}, $n=5$) but the cells responded to both nucleotides when they were administered in the reverse sequence (UDP: 37.2 ± 3.0 μA cm^{-2}, UTP: 35.9 ± 1.3 μA cm^{-2}, $n=5$). However, the increase in I_{SC} evoked by exposing UDP-stimulated epithelia to UTP was smaller than that seen in the experiments in which UTP was administered as the first agonist ($P<0.01$).

The interactions between ATP and UDP were also explored with this desensitization protocol. The epithelia responded to both nucleotides (both at 100 μM) whether they were first stimulated with UDP (33.8 ± 2.1 μA cm^{-2}, $n=8$) and then exposed to ATP (26.1 ± 0.1 μA cm^{-2}); or exposed to ATP (59.2 ± 6.4 μA cm^{-2}, $n=8$) and then UDP (22.8 ± 3 μA cm^{-2}). However, the responses seen when ATP and UDP were used as second agonists, were smaller ($P<0.01$ for both) than the responses evoked when these nucleotides were used as first agonists.

Discussion

The positive currents evoked by extracellular nucleotides appeared to be carried by anions moving from the basolateral compartment, across the epithelia and into the apical fluid. Such electrogenic anion transport is the process that underlies exocrine fluid secretion (reviewed by Petersen, 1992) and so our data confirm that nucleotides evoke secretory responses in epithelial cells (see e.g. Flezar & Heisler, 1993; Middleton *et al*., 1993; Martin & Shuttleworth, 1995). Although ATP and UTP evoked secretion when added to the apical or the basolateral solutions, the apical responses were always large and highly reproducible whereas the basolateral responses were very small and variable. The receptors underlying these responses therefore appear to be essentially confined to the apical plasma membrane (see also Wilson *et al*., 1996). Although it is essentially impossible for such apical purinoceptors to be activated by nucleotides released from periglandular nerves, there is a body of evidence to suggest such receptors are present in many polarized epithelia. Their physiological role remains enigmatic (Wong, 1988; Mason *et al*., 1991; Schwiebert *et al*., 1995; Xu *et al*., 1996).

The responses to apical nucleotides were transient and this was surprising as earlier studies of E/92/3 cells, grown as small groups on glass cover slips, showed that ATP and UTP could evoke sustained increases in $[Ca^{2+}]_i$ (Ko *et al*., 1994). Moreover, experiments in which these nucleotides were repeatedly added to the apical bath, showed that the rapid decline in I_{SC} was not due to the hydrolysis of these substances in the extracellular fluid. The cultured epithelia therefore appear to lose their sensitivity to nucleotides rapidly. Many agonists that increase $[Ca^{2+}]_i$, also activate a family of $[Ca^{2+}]_i$- and phospholipid-dependent enzymes known as the calcium-dependent protein kinases (PKC) (Berridge, 1993). These enzymes control many aspects of cellular physiology, but the increased activity of PKC that develops during prolonged or repeated stimulation causes a loss of sensitivity to the stimulating agonist (Llano & Marty, 1987; Maruyama, 1989). Earlier work showed that nucleotide-evoked calcium signals in E/92/3 cells were subjected to this PKC-dependent down regulation (Ko *et al*., 1994) and so we explored the possibility that this may explain the rapid desensitization seen in the cultured epithelia. TPA, an exogenous activator of PKC, lowered sensitivity to UTP, confirming that increased activity of PKC is associated with reduced sensitivity to calcium-mobilizing agonists (Llano & Marty, 1987; Maruyama, 1989). However, the present responses were unaffected by the PKC inhibitor staurosporine whereas our previous studies showed that this substance, at the

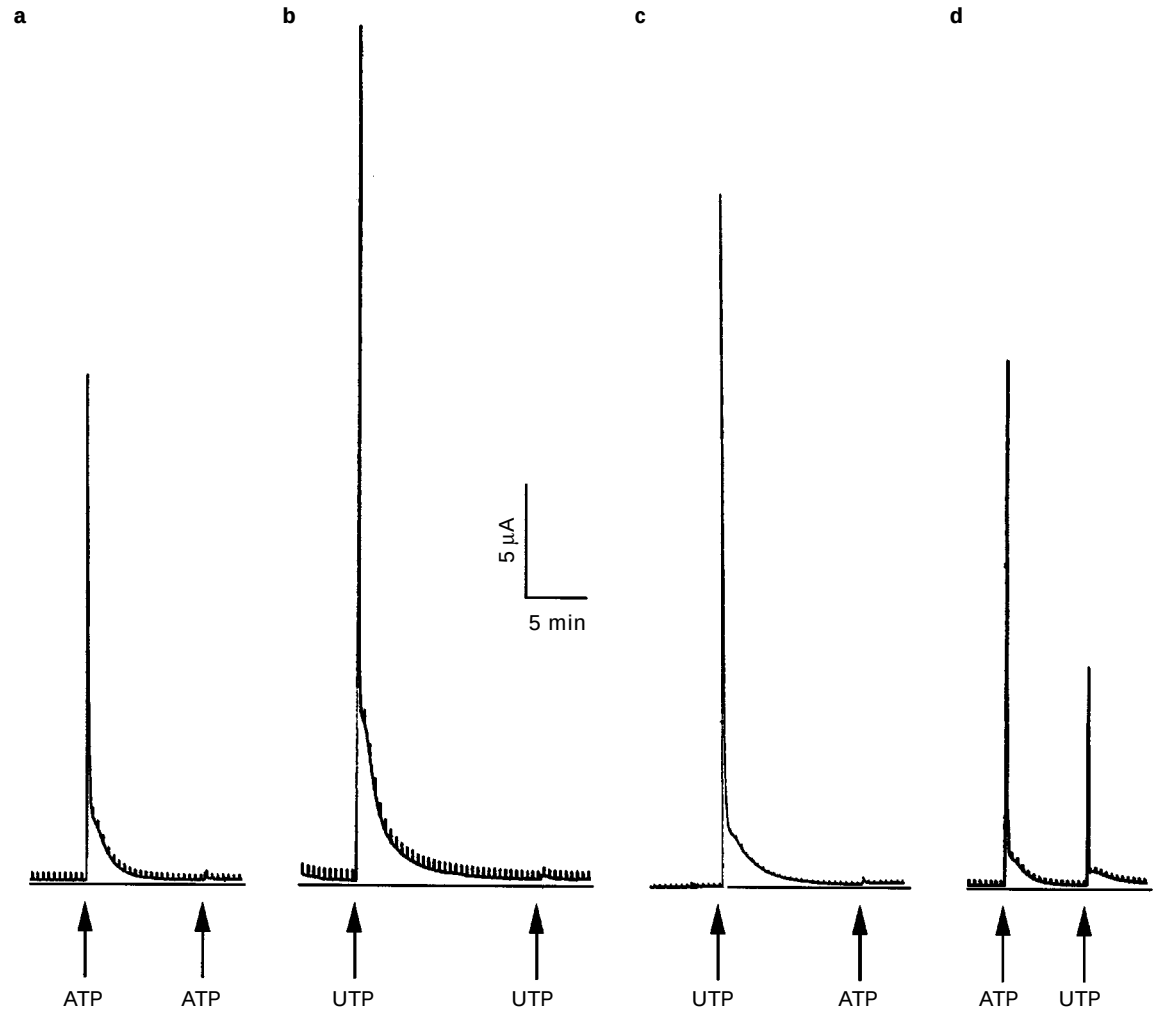

Figure 6 Desensitization of responses to apical nucleotides. Typical records showing (a) the effects of repeatedly adding aliquots of 100 μM ATP to the solution bathing the apical aspect of the cell layers ($n=5$); (b) the effects of repeatedly adding 100 μM UTP ($n=4$); (c) the effects of exposing epithelia to 100 μM UTP and then 100 μM ATP ($n=5$), and (d) the effects of stimulating cells with 100 μM ATP and then 100 μM UTP ($n=5$).

concentration used in the present experiments, could totally block the desensitization seen when either E/92/3 cells or aortic smooth muscle cells were repeatedly stimulated (Ko *et al*., 1994; Pediani *et al*., 1996). It is therefore clear that receptor-mediated activation of PKC does not underlie the rapid loss of sensitivity which we now describe.

The hormones and neurotransmitters which control fluid secretion generally increase the anionic permeability of the apical membrane and the potassium permeability of the basolateral membrane. The rise in anion permeability facilitates the diffusion of anions across the apical membrane, whereas the increased potassium permeability hyperpolarizes the cell and so increases the driving force for this efflux of anions into the glandular lumen (reviewed by Petersen, 1992), Earlier studies of E/92/3 cells in monolayer culture showed that thapsigargin, which increases $[Ca^{2+}]_i$ by inhibiting the calcium-pumping enzymes of the endoplasmic reticulum, also evoked anion secretion. Moreover, this response was sustained for a long period of time but was abolished when barium, a cation that blocks potassium channels, was added to the basolateral solution (Ko *et al*., 1996). However, the present responses to nucleotides, were only slightly reduced by barium. It therefore appears that increased potassium channel activity makes only a minor contribution to this response. It is interesting, in this context, that isotope efflux experiments showed that UTP and ATP caused only transient increases in membrane anion permeability. We therefore suggested that, once activated, the $[Ca^{2+}]_i$-dependent anion channels expressed by these cells may rapidly lose their sensitivity to calcium (Wilson *et al*., 1995). It is possible that this may also account for the transient nature of the present responses.

P_{2U} receptors belong to a receptor family whose structure is defined by seven, membrane-spanning polypeptide domains (Dubyak & El-Moatassim, 1993; Lustig *et al*., 1993; Parr *et al*., 1994). When activated, all such receptors interact with membrane proteins, known as guanine nucleotide-binding proteins (G proteins), that transmit signals across the plasma membrane (see review by Sternweis & Smrcka, 1992). P_{2U} receptors can be activated, with equal efficacy and potency, by either ATP or UTP and these receptors appear to be linked to their intracellular effectors by a G protein family that is inactivated by pertussis toxin (Dubyak & El-Moatassim, 1993; Parr *et al*., 1994). The results of earlier studies were, therefore, entirely consistent with the view that it was P_{2U} receptors which allowed extracellular nucleotides to increase $[Ca^{2+}]_i$ in E/92/3 cells (Ko *et al*., 1994; Wilson *et al*., 1996). Although the present responses to UTP and ATP were reduced by pertussis toxin, the epithelia were ca 13 times more sensitive to UTP than to ATP and this is not consistent with the hypothesis that responses to these nucleotides are simply mediated via P_{2U} receptors (Ko *et al*., 1994; Wilson *et al*., 1996). This result thus raised the possibility that the rise in $[Ca^{2+}]_i$ and the increase in I_{SC} may have different pharmacological bases.

Studies of other epithelia showed that apical purinoceptors can allow nucleotides to activate anion channels by a mechanism that does not involve a cytoplasmic second messenger signal (Stutts *et al*., 1994; Guo *et al*., 1995; Schwiebert *et al*., 1995). We therefore explored the extent to which the present responses were dependent upon $[Ca^{2+}]_i$ by loading epithelia with BAPTA, a substance that abolishes $[Ca^{2+}]_i$-dependent responses by artificially increasing the cytoplasm's Ca^{2+}-buffering capacity. BAPTA inhibited, but did not entirely eliminate, the responses to ATP and UTP. Separate experiments, undertaken with cells grown on coverslips, confirmed that this calcium chelator totally abolished the rise in $[Ca^{2+}]_i$ evoked by 100 μM UTP (J.D. Pediani and S.M. Wilson, unpublished). Part of the responses to ATP and UTP therefore appears to be mediated via a mechanism that is independent of increased $[Ca^{2+}]_i$. However, analysis of these data showed that BAPTA reduced the response to UTP more than the response to ATP, and this differential sensitivity was not consistent with the view that these nucleotides act via a common, P_{2U} receptor. Experiments undertaken with a classical, cross-desensitization protocol, showed that the apical membrane contained at least two populations of UTP-sensitive receptor. Whilst one of these appeared to be sensitive to both ATP and UTP, the other was selectively activated by UTP. Experiments with pertussis toxin-treated epithelia indicated that these receptor populations were equally sensitive to this toxin, suggesting that both couple to pertussis toxin-sensitive G proteins. Subsequent work showed that apical UDP also increase I_{SC} and suggested that this nucleotide may selectively activate the ATP-insensitive receptors population. UDP may, therefore, become a useful agonist with which to explore the cellular signal transduction pathways that can be controlled via the ATP-insensitive receptor population.

The P_{2U} receptor previously described in these cells (Ko *et al*., 1994; Wilson *et al*., 1996) may well account for the receptor population sensitive to ATP and UTP, but it is not immediately apparent what molecular species may underlie the ATP-insensitive receptor population. However, the existence of receptors sensitive to pyrimidine nucleotides, but not purine nucleotides (i.e. pyrimidinoceptors) was proposed almost twenty years ago (Urquilla, 1978). Since then, evidence supporting the hypothesis has emerged from functional studies of several non-epithelial cell types (see e.g. Seifert & Schulz, 1989; Klügelgen *et al*., 1990; Chinellato *et al*., 1994; Connolly, 1994; Johnson & Hourani, 1994; Lazarowski & Harden, 1994; Boehn *et al*., 1995; Garcia-Velasco *et al*., 1995; Yang *et al*., 1996). More recently, genes encoding two previously undescribed, G-protein activating receptors have been cloned (Chang *et al*., 1995; Communi *et al*., 1995; Nguyen *et al*., 1995). These novel receptors have close homology with the P_{2U} receptor and, whilst both are sensitive to UTP, one appears to be totally insensitive to purine nucleotides (Communi *et al*., 1995; Nguyen *et al*., 1995) whereas the other is activated only by extremely high concentrations of ATP (Chang *et al*., 1995). These molecular studies have, therefore, unequivocally established the existence of pyrimidinoceptors.

The present data suggest strongly that pyrimidinoceptors are present in the apical membranes of E/92/3 cells, and we believe that this is the first indication that such receptors are expressed by epithelia. Their apical location makes their physiological role hard to infer, but it has been proposed that ATP, and possibly other nucleotides, may be released across the apical membranes of stimulated epithelia (Reisin *et al*., 1994). The nucleotides released in this way could activate the apical purinoceptors and so act as autocrine regulators of epithelial transport (Parr *et al*., 1994; Schwiebert *et al*., 1995; Wilson *et al*., 1996). Our suggestion that pyrimidinoceptors are also present in this membrane therefore raises the possibility that pyrimidine nucleotides may also be involved in such autocrine regulation of epithelial function.

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