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ACTIVATION OF APICAL P_{2U} PURINE RECEPTORS PERMITS INHIBITION OF ADRENALINE-EVOKED CYCLIC AMP ACCUMULATION IN CULTURED EQUINE SWEAT GLAND EPITHELIAL CELLS

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Summary

Experiments were undertaken using cultured equine sweat gland epithelial cells that express purine receptors belonging to the P_{2U} subclass which allow the selective agonist uridine triphosphate (UTP) to increase the concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$). Experiments using pertussis toxin (Ptx), which inactivates certain guanine-nucleotide-binding proteins (G-proteins), showed that this response consisted of Ptx-sensitive and Ptx-resistant components, and immunochemical analyses of the G-protein α subunits present in the cells showed that both Ptx-sensitive (αi1–3) and Ptx-resistant (αq/11) G-proteins were expressed. P_{2U} receptors may, therefore, normally activate both of these G-protein families. Ptx-sensitive, αi2/3 subunits permit inhibitory control of adenylate cyclase, and UTP was shown to cause Ptx-sensitive inhibition of adrenaline-evoked cyclic AMP accumulation, suggesting that the receptors activate $G_{i2/3}$. Experiments using cells grown on permeable supports suggested that P_{2U} receptors became essentially confined to the apical membrane in post-confluent cultures. Polarised epithelia may, therefore, express apical P_{2U} receptors which influence two centrally important signal transduction pathways. It is highly improbable that these receptors could be activated by nucleotides released from purinergic nerves, but they may be involved in the autocrine regulation of epithelial function.

Key words: G-proteins, pertussis toxin, cell signalling, nucleotide receptors, sweat glands, intracellular free Ca^{2+} concentration.

Introduction

Regulated changes to the anionic conductance of the apical plasma membrane are fundamentally important to the control of transepithelial fluid and electrolyte transport, and secretagogues generally exert this control by binding to basolateral receptors and evoking second-messenger signals which influence the activity of anion channels in the apical plasma membrane (e.g. Cliff and Frizzell, 1990). 'Ca^{2+}-mobilising' agonists, such as acetylcholine, bind to receptors that are functionally coupled to phospholipase C (PLC) and they can therefore increase both the concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) and the activity of protein kinase C (PKC) (Berridge, 1993). Agonists such as adrenaline and noradrenaline, however, can also evoke cyclic adenosine 3′,5′-monophosphate (cyclic AMP) formation by binding to separate receptors that permit control over adenylate cyclase (Levitzki, 1988). However, as well as their receptors for these classical autonomic agonists, many epithelia express purine receptors belonging to the P_{2U} subclass that allow extracellular ATP, or the more selective agonist uridine triphosphate (UTP), to increase $[Ca^{2+}]_i$. Although these receptors are widespread, their physiological significance is not obvious (Dubyak and El-Moatassim, 1993).

The P_{2U} receptor belongs to a structurally homologous group of receptors characterised by seven membrane-spanning domains (Dubyak and El-Moatassim, 1993; Parr *et al.* 1994). This receptor family allows hormones and neurotransmitters to regulate intracellular effector enzymes because its members are all able to interact with a group of heterotrimeric membrane proteins known collectively as the guanine-nucleotide-binding proteins (G-proteins) (Sternweis and Smrcka, 1992). In unstimulated cells, G-proteins are characteristically found in their heterotrimeric configuration, but they dissociate into two subunits, the GTP-bearing α subunit and the dimeric βγ subunit, once they interact with activated receptors. Both of these subunits are biologically active, but there are many structurally and functionally distinct isoforms of the α subunit.

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In many cell types, P_{2U}-receptor-mediated responses are antagonised by pertussis toxin (Ptx) (Cockcroft and Stutchfield, 1989; Dubyak and El-Moatassim, 1993), a toxin that catalyses the ADP-ribosylation of α subunits belonging to the αi and αo families; G-proteins containing such Ptx-modified subunits (G_i and G_o, respectively) become locked into the heterotrimeric configuration and can no longer play a role in transmembrane signalling (Katada and Ui, 1982; Sternweis and Smrcka, 1992). Ptx does not, however, affect the responses to several other Ca^{2+}-mobilising agonists (Berridge, 1993), and their sensitivity to Ptx therefore suggests that P_{2U} receptors interact with a different signal transduction pathway from most Ca^{2+}-mobilising receptors (Berridge, 1993; Dubyak and El-Moatassim, 1993). Elucidation of the pathways that can be controlled by P_{2U} receptors may clarify their physiological role and so we have explored the effects of Ptx upon UTP-evoked second-messenger signals in an epithelial cell line derived from the equine sweat gland (Wilson *et al.* 1993; Ko *et al.* 1994*a*). Some of the data have been presented to the Physiological Society (Rakhit *et al.* 1994, 1995).

Materials and methods

Most methods are described elsewhere (Wong, 1988; Wilson *et al.* 1993; Ko *et al.* 1994*a*) and so only brief details are presented here. Standard techniques were used to maintain a spontaneously transformed, epithelial cell line derived from the equine sweat gland in William's medium E supplemented as detailed previously (Wilson *et al.* 1993). For experiments, cells were removed from the culture flasks using trypsin/EDTA and plated onto glass coverslips, 24-well plates or permeable supports fabricated from Millipore filters. Ptx-treated cells were prepared by incubating cells in medium containing this toxin (10 ng ml^{-1}) for 18–24 h immediately prior to each experiment.

Measurement of intracellular free Ca^{2+} levels

Cells plated onto glass coverslips were loaded with the Ca^{2+}-sensitive fluorescent dye Fura-2 (Grynkiewicz *et al.* 1985) by incubation (30–40 min) in medium containing the dye's membrane-permeant acetoxymethyl ester form. The coverslips were then mounted in a small chamber attached to the stage of an inverted microscope, where the cells were superfused (at 37 °C) with physiological saline containing (in mmol l^{-1}): NaCl, 130; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1; Hepes, 20; and D-glucose, 10 (pH adjusted to 7.4 with NaOH). Fura-2 fluorescence ratios (excitation wavelengths 340 and 380 nm) were recorded from groups of 2–5 cells. Responses were quantified by measuring the Fura-2 fluorescence ratio at the peak of a response and subtracting from it the ratio measured immediately prior to application of UTP. Values of *N* refer to the number of experiments in each group.

Assay of cellular cyclic AMP content

Cells grown (2–3 days) on 24-well plates were washed with physiological saline containing 20 μmol l^{-1} papaverine, a phosphodiesterase inhibitor included to inhibit cyclic AMP hydrolysis, and briefly incubated (at 37 °C) in this solution until experiments were initiated by addition of agonists. After 10 min, concentrated perchloric acid was added to each well (final concentration 0.1 mol l^{-1}) to arrest cellular metabolism and extract cyclic AMP. The acid extracts were recovered, neutralised and their cyclic AMP contents determined by radioimmunoassay (Ko *et al.* 1994*a*). Cellular protein was also extracted from each well using 1 % sodium dodecyl sulphate (SDS) in 0.3 mol l^{-1} NaOH, assayed and data were expressed as picomoles of cyclic AMP per microgram of cellular protein (pmol μg^{-1}). Increases in cyclic AMP content were quantified by subtracting the cyclic AMP content of unstimulated cells from the cyclic AMP content of stimulated cells grown on the same plates. Determinations were made in duplicate or triplicate, and values of *N* refer to the number of times a protocol was repeated using cells at different passage number.

Detection of G-protein α subunit isoforms

Cells harvested by scraping into 250 mmol l^{-1} sucrose solution containing DNAase I (4 μg ml^{-1}), protease inhibitors (52 μg ml^{-1} apoprotin; 20 μg ml^{-1} leupeptin; 20 μg ml^{-1} pepstatin; 35 μg ml^{-1} phenylmethylsulphonyl fluoride, PMSF) and 10 mmol l^{-1} triethanolamine/HCl (pH 7.6) were disrupted by homogenisation, and membrane proteins were prepared from the homogenate by differential centrifugation. Samples of protein (30–40 μg) were fractionated by SDS–polyacrylamide gel electrophoresis. Standard gels consisted of 10 % acrylamide/0.26 % bisacrylamide. However, several G-protein α subunit isoforms have essentially identical mobilities on such gels because their molecular masses are very similar. Some analyses were therefore undertaken by fractionating proteins on urea-containing gels (12.5 % acrylamide/0.065 % bisacrylamide, 6 mol l^{-1} urea) as even closely related proteins can become differentially mobile under these conditions (Milligan *et al.* 1989). Fractionated proteins were blotted onto nitrocellulose membranes by semi-dry electrophoresis and the membranes were immunostained using antisera against amino acid sequences specific to defined α subunit isoforms (Milligan *et al.* 1989). Positive bands were visualised by enhanced chemiluminescence (Amersham ECL kit) using a peroxidase-conjugated second antibody against rabbit IgG. The efficacy of the fractionation and staining procedures was confirmed using membrane proteins isolated from either guinea pig brain or mouse lung.

Measurement of short-circuit current (I_{sc})

Cells (approximately 100 000) were seeded onto permeable supports (0.45 cm^2) and the formation of a coherent layer was monitored by measuring transepithelial resistance, which fell to a stable value of approximately 300 Ω cm^2 after 4–5 days. Epithelial cells grown in this manner can develop a polarised morphology in which the upward-facing aspect of the cell layer is equivalent to the apical surface (e.g. Wong, 1988). Filter assemblies bearing such layers were mounted in Ussing chambers designed to allow drugs to be added selectively to the solutions bathing either the apical or basolateral aspects of

the layer. The current required to clamp the transepithelial potential difference to 0 mV (short-circuit current, I_{sc}) was monitored and displayed continuously. The standard physiological saline contained (in μmol l^{-1}) NaCl, 117; $NaHCO_3$, 25; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; D-glucose, 11.1 (pH 7.4 when gassed with 95 % O_2/5 % CO_2). Chloride- and bicarbonate-free solutions were prepared by replacing NaCl, KCl and $CaCl_2$ with the appropriate gluconate salts and CO_2/$NaHCO_3$ with Hepes/NaOH.

Results

Effects of Ptx upon UTP-evoked Ca^{2+} signals

A series of pulses (30 s) of increasing UTP concentration each evoked a rise in $[Ca^{2+}]_i$ (Fura-2 fluorescence ratio) that fell back to control levels once superfusion was switched back to the control solution (Fig. 1A). This response, which has been described previously, is attributable to P_{2U}-receptor-mediated activation of PLC (Dubyak and El-Moatassim, 1993; Ko *et al.* 1994*a*). To assess the contribution that Ptx-sensitive G-proteins make to this response, we explored the effects of Ptx upon these Ca^{2+} signals. UTP evoked a concentration-dependent increase in $[Ca^{2+}]_i$ in both control and Ptx-treated cells, but analysis of variance showed that the data obtained from the two groups did not belong to the same statistical population (P<0.05), and further analysis (Bonferroni multiple-comparison test) indicated that the responses to the highest concentrations of UTP tested in the Ptx-treated cells were smaller than control responses (Fig. 1B). The significance of this result was confirmed using Student's *t*-test. Ptx therefore attenuates the responses to maximally effective concentrations of UTP but does not affect the responses to lower concentrations (Fig. 1B).

Effects of UTP upon adrenaline-evoked accumulation of cyclic AMP

We explored the effects of UTP upon adrenaline-evoked cyclic AMP accumulation by quantifying adrenaline-evoked increases in cyclic AMP content under control conditions and in the presence of UTP (0.01–1 mmol l^{-1}). The cyclic AMP content of unstimulated cells was normally 0.137±0.034 pmol μg^{-1} (mean ± S.E.M.; N=11) and, in these experiments, increased approximately sixfold during stimulation with 10 μmol l^{-1} adrenaline. Earlier work indicated that this response was attributable to β_2-adrenoceptor-mediated activation of adenylate cyclase (Wilson *et al.* 1993). Adrenaline also evoked cyclic AMP accumulation in the presence of UTP, but these responses were smaller than the control value and this effect was statistically significant for UTP concentrations of 0.1 mmol l^{-1} and above (Fig. 2A). The inhibitory actions of these effective concentrations of UTP were therefore quantified and are presented in Fig. 2B together with equivalent data from Ptx-treated cells. This toxin did not (mean ± S.E.M.; N=11) affect basal cyclic AMP content (0.115±0.026 pmol μg^{-1}) or the magnitude of the response to adrenaline (0.762±0.183 pmol μg^{-1}) but essentially abolished the inhibitory action of UTP (Fig. 2B).

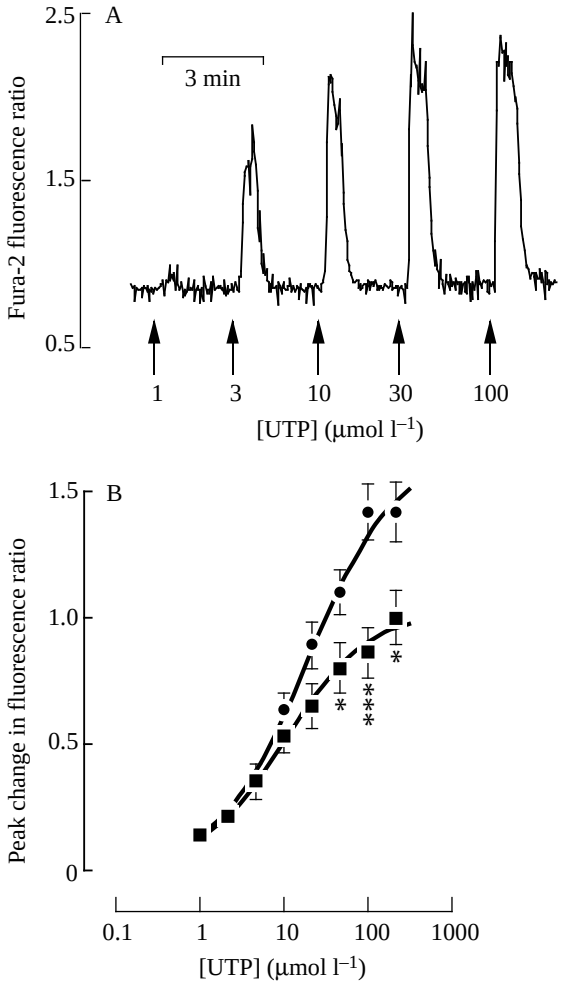

Fig. 1. Effects of pertussis toxin (Ptx) upon P_{2U}-receptor-mediated increases in $[Ca^{2+}]_i$. (A) A typical record obtained during an experiment in which cells were repeatedly stimulated with 30 s pulses of UTP. (B) UTP-evoked increases in Fura-2 fluorescence ratio were quantified and plotted (mean ± S.E.M.) against the concentration of UTP used. Sigmoid curves were fitted to the experimental data using a least-squared regression procedure; ●, data from control cells (N=10); ■, data from Ptx-treated cells at identical passage number (N=10). Asterisks denote data points that differed significantly from the appropriate control values (*P<0.05, ***P<0.01).

We used a strictly paired experimental protocol to explore the possibility that the UTP-evoked rise in $[Ca^{2+}]_i$ may underlie this inhibition. In these experiments (N=27), the cyclic AMP content of unstimulated control cells was 0.040±0.005 pmol μg^{-1} and normally increased by 0.615±0.095 pmol μg^{-1} during exposure to adrenaline (10 μmol l^{-1}). Removing external Ca^{2+} increased the cyclic AMP content of unstimulated cells (P<0.02, Student's paired *t*-test), but none of the experimental manoeuvres affected the magnitude of the response to adrenaline, establishing that the procedures did not affected the cell's capacity to synthesise cyclic AMP *per se*. Removing Ca^{2+} from the external saline

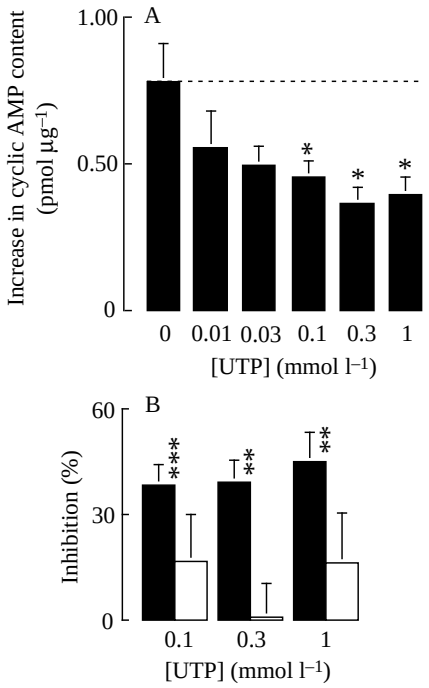

Fig. 2. Effects of UTP upon adrenaline-evoked cyclic AMP accumulation. (A) Responses to 10 μmol l^{-1} adrenaline were quantified under control conditions (dashed line) and in the presence of UTP. Data from 11 experiments are presented, but not all concentrations of UTP were tested in each experiment and so each value is the mean + S.E.M. of between 4 and 11 independent observations. Data were analysed using analysis of variance/Bonferroni multiple-comparison tests, and asterisks denote values that differed significantly from the control value (P<0.05). The significance of this analysis was confirmed using Student's t-tests. (B) The inhibitory action of the effective concentrations of UTP was quantified by comparison with the responses seen in control cells grown on the same plates. These data (filled columns) are presented with equivalent data from pertussis toxin (Ptx)-treated cells at identical passage number (open columns). Asterisks denote the significance of the inhibitory action (Student's t-test); **P<0.02, ***P<0.001.

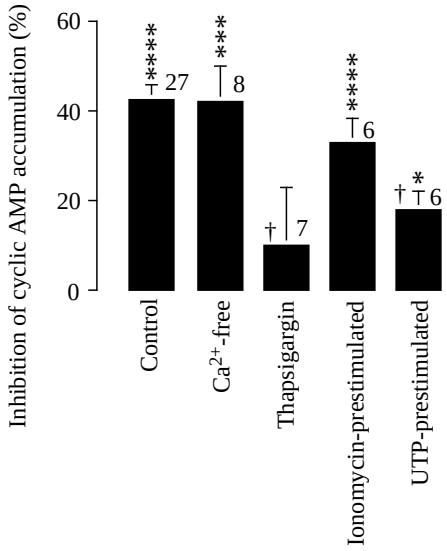

Fig. 3. The effects of manipulations that impair the P_{2U}-receptor-mediated increase in $[Ca^{2+}]_i$ upon UTP-evoked inhibition of cyclic AMP accumulation. In each experiment, we measured the cyclic AMP contents of unstimulated cells, of cells that had been incubated in 10 μmol l^{-1} adrenaline and of cells that had been incubated in both 10 μmol l^{-1} adrenaline and 1 mmol l^{-1} UTP. Each such measurement was repeated for control cells and for experimental cells grown on the same plates that had been subjected to one of the listed experimental manipulations. These data were used to obtain paired estimates of the inhibitory effect of UTP, analysed using Student's paired t-tests and presented as percentage inhibition (mean + S.E.M.); values of N are given beside each column. Thapsigargin-treated cells were incubated in Ca^{2+}-free saline containing this substance (0.1 μmol l^{-1}) for 5 min before experiments were initiated by addition of agonists to the appropriate wells. Ionomycin-prestimulated cells were exposed to Ca^{2+}-free saline containing this ionophore (0.1 μmol l^{-1}) for 5 min, washed with Ca^{2+}-free saline and then incubated in Ca^{2+}-free saline for 15 min until experiments were initiated by addition of agonists. UTP-prestimulated cells were exposed to Ca^{2+}-free saline containing UTP (100 μmol l^{-1}) for 2 min, then maintained in under Ca^{2+}-free conditions for 15 min before experiments were initiated. Asterisks denote experiments in which UTP caused significant inhibition of cyclic AMP accumulation (*P<0.02, ***P<0.005, ****P<0.002), and daggers (†) indicate experiments in which the inhibitory effect of UTP differed significantly from that measured in control cells grown on the same plates (P<0.02).

did not affect the inhibitory action of UTP (Fig. 3), establishing that it is not mediated by Ca^{2+} entering the cell *via* the P_{2U}-receptor-regulated influx pathway (Dubyak and El-Moatassim, 1993; Ko *et al.* 1994*a*). In order to explore the possibility that the UTP-evoked release of Ca^{2+} from internal stores (Dubyak and El-Moatassim, 1993; Ko *et al.* 1994*a*) may contribute to the inhibition, cells were deprived of external Ca^{2+} and subjected to procedures designed to compromise the sequestration of Ca^{2+} within agonist-sensitive stores. In the first such experiments, cells were exposed to Ca^{2+}-free saline containing 0.1 μmol l^{-1} thapsigargin for 5 min before experiments were initiated by addition of agonists. This essentially abolished the inhibitory action of UTP (Fig. 3). In further experiments, the agonist-sensitive internal stores were depleted by incubating cells in Ca^{2+}-free saline containing ionomycin (0.1 μmol l^{-1}, 5 min) or UTP (100 μmol l^{-1}, 2 min). The cells were then washed with Ca^{2+}-free saline and incubated in this solution for a further 15 min until agonists were added to the appropriate wells. Significant inhibition of adrenaline-evoked cyclic AMP accumulation occurred in both groups, although the effect was smaller than control values after prestimulation with UTP (Fig. 3).

P_{2U} receptor agonists may activate PKC as well as increasing $[Ca^{2+}]_i$ (Dubyak and El-Moatassim, 1993) and we explored the possibility that this may contribute to their inhibitory action (e.g. Murphy *et al.* 1987) by incubating cells in saline containing 0.16 μmol l^{-1} 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) or 0.1 μmol l^{-1} staurosporine for 5 min before experiments were initiated. These substances activate and

inhibit PKC, respectively, although staurosporine can also inhibit other kinases. Neither agent affected basal cyclic AMP content or the magnitude of the response to adrenaline. Moreover, UTP (1 mmol l^{-1}) caused normal inhibition of cyclic AMP accumulation in the presence of TPA (control, 40.2±3.7 %; TPA, 44.1±4.5 %; N=5; means ± S.E.M.) but became a more effective inhibitor after exposure to staurosporine (control, 41.2±7.3 %; staurosporine, 65.7±5.3 %; N=4, P<0.05, Student's paired t-test).

Expression of G-protein α subunit isoforms

Antisera against the N-terminal or C-terminal decapeptide of αo (ON1 and OC1, respectively, Milligan *et al.* 1989) produced a positive reaction to membrane proteins isolated from guinea pig brain but no reaction to proteins isolated from the equine cells (Fig. 4A,B). Two bands were detected when proteins isolated from either mouse lung or the equine cells were fractionated on urea-containing gels and stained using a primary antiserum (SG1, Milligan *et al.* 1989) against a sequence present in both αi1 and αi2 (Fig. 4C). Further analyses (N=3) showed that the more mobile of these bands co-migrated with a recombinant αi2 standard (Calbiochem 371759-S). A primary antiserum against an amino acid sequence unique to αi3 (Calbiochem 371729-S) produced a single positive band when proteins isolated from guinea pig brain, mouse lung or the equine cells were fractionated on standard gels, although the signal from the equine cells was very weak. An antibody directed against an amino acid sequence common to αq and α11 (Calbiochem 371751-S) revealed two bands when membrane proteins from mouse lung were fractionated on urea-containing gels. However, when equine proteins were fractionated under these conditions, only a single positive band was detected and this did not co-migrate with either mouse lung isoform (Fig. 4D). The αq and α11 subunits are almost invariably co-expressed (Mitchell *et al.* 1991), and so the equine cells may be an unusual example of a cell type expressing only a single αq/11 isoform. However, we cannot specifically exclude the possibility that a small difference between the primary structures of the equine and rodent αq/11 families may confer anomalous mobility upon the equine isoforms and prevent their separation on urea-containing gels.

Regulation of transepithelial anion transport

We used Ussing chambers to explore the effects of UTP upon the transport of ions across cultures grown on permeable supports. Post-confluent cultures normally displayed a spontaneous transepithelial potential difference of 0.42±0.04 mV (apical side negative) and a basal I_{sc} of approximately 0.6 μA cm^{-2}. UTP increased I_{sc} when added to the solution bathing either side of the epithelium, and both responses consisted of an initial rapid rise followed by a decline towards basal levels (Fig. 5). These responses were essentially abolished by removing chloride and bicarbonate from the external solution (N=4), demonstrating that they are due to the transport of these anions from the basolateral to the apical compartments. However, the response evoked by exposing the basal side of the epithelium to UTP (ΔI_{sc}=3.2±0.5 μA cm^{-2}, N=6) was much smaller than that evoked by adding this nucleotide to the apical bath (ΔI_{sc}=71.7±6.4 μA cm^{-2}, N=6) (Fig. 5). The data therefore suggest that the P_{2U} receptors become essentially confined to the apical aspect of the cell layer in post-confluent cultures.

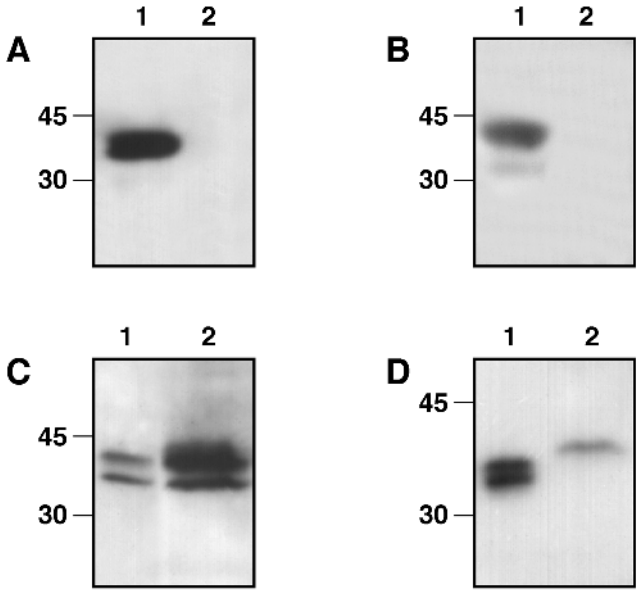

Fig. 4. Expression of G-protein α subunit isoforms. Nitrocellulose membranes bearing fractionated proteins were stained using antisera against (A) the N-terminal decapeptide of αo (ON1, Milligan *et al.* 1989), (B) the C-terminal decapeptide of αo (OC1, Milligan *et al.* 1989), (C) an amino acid sequence that is conserved between αi1 and αi2 (SG1, Milligan *et al.* 1989) and (D) an amino acid sequence common to αq and α11 (Calbiochem 371751-S). In each instance, appropriate positive control samples are in lane 1 whilst proteins extracted from equine cells are in lane 2. The bars indicate the positions of proteins of known molecular mass (kDa), although the proteins stained in C and D were fractionated on urea-containing gels and electrophoretic mobility is not a simple function of molecular mass under these conditions.

Discussion

P_{2U} receptor agonists characteristically increase $[Ca^{2+}]_i$ by activating PLC (Dubyak and El-Moatassim, 1993; Ko *et al.* 1994*a*). The present experiments, in common with many earlier studies (e.g. Dubyak and El-Moatassim, 1993; Parr *et al.* 1994), showed that this response consists of Ptx-sensitive and Ptx-resistant components, and our data therefore indicate that at least part of the response to UTP is mediated by G-proteins sensitive to this toxin. The only Ptx-sensitive G-protein α subunits that could be unambiguously detected in the equine cells were αi1 and αi2, although barely discernible levels of αi3 were also present. This pattern of G-protein expression, together with the sensitivity to Ptx, is consistent with the view that P_{2U} receptors normally interact with $G_{i2/3}$

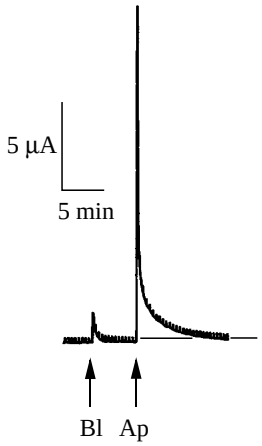

Fig. 5. Effects of UTP upon short-circuit current in cells grown on permeable supports. UTP (final concentration 100 μmol l^{-1}) was added to the solution bathing the basolateral (Bl) or apical (Ap) aspects of the epithelium as indicated by the arrows. Positive currents are defined as those carried by anions moving from the basal to the apical compartments and are shown as upward deflections of the trace. Essentially identical results were obtained in six instances.

(Dubyak and El-Moatassim, 1993). There was, however, a substantial toxin-resistant component to the increase in $[Ca^{2+}]_i$, which suggests that P_{2U} receptors also activate PLC *via* a Ptx-resistant pathway. At least one member of the Ptx-resistant $G_{q/11}$ family was present, and these G-proteins activate PLC in essentially all cell types (Berridge, 1993). It is therefore possible that P_{2U} receptors may normally increase $[Ca^{2+}]_i$ by activating both $G_{i2/3}$ and $G_{q/11}$. Such interactions between a single receptor and multiple G-proteins have been documented in other systems (reviewed by Kenakin, 1995*a*,*b*).

Although both $G_{i2/3}$ and $G_{q/11}$ allow activated receptors to increase $[Ca^{2+}]_i$, they do so by different mechanisms. It is the $\beta\gamma$ subunit released from $G_{i2/3}$ that activates the cytosolic β_2 isoform of PLC (PLC-β_2), whereas it is the α subunit released from $G_{q/11}$ that activates the membrane-bound β_1 isoform of this enzyme (Camps *et al.* 1992; Katz *et al.* 1992; Berridge, 1993; Clapham and Neer, 1993). However, the most important difference between these two pathways is that α subunits released from $G_{i2/3}$ inhibit adenylate cyclase; receptors that couple to $G_{i2/3}$ therefore allow agonists to inhibit cyclic AMP formation as well to increase $[Ca^{2+}]_i$ (Simonds *et al.* 1989; McKenzie and Milligan, 1990). The suggestion that the P_{2U}-receptor-mediated rise in $[Ca^{2+}]_i$ is mediated, at least in part, by $G_{i2/3}$ therefore raised the possibility that this response may be accompanied by inhibition of adenylate cyclase, and our data showed clearly that UTP consistently inhibited adrenaline-evoked cyclic AMP accumulation. This agrees with recent studies of smooth muscle cells (Sipma *et al.* 1994) and renal mesangial cells (Schulze-Lohoff *et al.* 1995), and with the data from earlier studies which showed that ATP could antagonise cyclic AMP accumulation in several cell types (reviewed by Dubyak and El-Moatassim, 1993), including hepatocytes and thyroid cells (Okajima *et al.* 1987; Sato *et al.* 1992). The receptor subtype underlying this inhibitory action was not identified in these early studies, but P_{2U} receptors have since been identified in many of the cell types in which the action was described (Dubyak and El-Moatassim, 1993). P_{2U} receptors may, therefore, provide a widespread mechanism that allows extracellular nucleotides to antagonise cyclic AMP accumulation.

Most earlier data suggested that this inhibition was due to an inhibitory interaction between $\alpha i2/3$ subunits and adenylate cyclase (Okajima *et al.* 1987; Sato *et al.* 1992; Dubyak and El-Moatassim, 1993; Sipma *et al.* 1994; Schulze-Lohoff *et al.* 1995). Whilst the sensitivity to Ptx which we now report is consistent with this mechanism, earlier work showed that ionomycin inhibited cyclic AMP accumulation in these cells (Wilson *et al.* 1993). This suggested that large increases in $[Ca^{2+}]_i$ may inhibit cyclic AMP accumulation, and the present data show that the UTP-evoked rise in $[Ca^{2+}]_i$ and inhibition of cyclic AMP accumulation are both sensitive to Ptx. We therefore undertook experiments to explore the possibility that the inhibition may be due simply to increased $[Ca^{2+}]_i$. Removing external Ca^{2+} did not affect the inhibitory action, establishing that it was not mediated by Ca^{2+} entering the cell *via* the P_{2U}-receptor-regulated influx pathway. Whilst this agrees well with the earlier studies (Okajima *et al.* 1987; Sato *et al.* 1992; Dubyak and El-Moatassim, 1993; Sipma *et al.* 1994; Schulze-Lohoff *et al.* 1995), P_{2U} receptor agonists also evoke the release of Ca^{2+} from a cytoplasmic store. UTP therefore causes a transient rise in $[Ca^{2+}]_i$ even if external Ca^{2+} is absent (Dubyak and El-Moatassim, 1993; Ko *et al.* 1994*a*), and earlier studies do not appear to have directly addressed the possibility that this may contribute to its inhibitory action (Okajima *et al.* 1987; Sato *et al.* 1992; Dubyak and El-Moatassim, 1993; Sipma *et al.* 1994). We therefore explored the effects of a number of experimental approaches designed to deplete the cytoplasmic Ca^{2+} store: exposure to thapsigargin, prestimulation with ionomycin and prestimulation with UTP. Earlier work had established that thapsigargin allows Ca^{2+} to drain from this internal store and showed that, under Ca^{2+}-free conditions, the P_{2U} receptor agonist cannot increase $[Ca^{2+}]_i$ in thapsigargin-treated cells (Ko *et al.* 1994*b*). The conditions required to deplete these stores using ionomycin or UTP had also been determined in previous studies (Ko *et al.* 1994*a*, 1995) and, in the present experiments, cells were allowed to recover from prestimulation with these agents in Ca^{2+}-free saline so that there could be no refilling of the Ca^{2+} store. These experiments showed that the inhibition was abolished by thapsigargin but unaffected by ionomycin. Furthermore, although significant inhibition persisted after prestimulation with UTP, this effect was smaller than control levels of inhibition. Although these experiments did not provide a consistent picture, we believe that, on balance, most of the available data suggest that the inhibition is not due to increased $[Ca^{2+}]_i$. However, at least some of our experiments suggest that activation of the Ptx-sensitive inhibitory pathway requires Ca^{2+} to be present within the internal store. The physiological basis of this effect has not been established.

The data showed clearly that the inhibition was not mediated

by PKC; indeed, they suggest that tonic activity of a kinase, although probably not PKC, may normally limit the inhibitory action of UTP. It is interesting, however, that the inhibitory action was entirely normal after 5 min of incubation in TPA, whereas P_{2U}-receptor-mediated Ca^{2+} signals attenuated rapidly in the presence of this substance (Ko *et al.* 1994*a*). The rise in $[Ca^{2+}]_i$ and the inhibition of cyclic AMP accumulation therefore appear to be differentially sensitive to regulation *via* PKC, suggesting that different signalling pathways underlie these events.

Earlier studies of the cells used in the present study, and of other epithelial cell types, showed that P_{2U} receptors allowed extracellular nucleotides to evoke changes in membrane permeability that were highly suggestive of a secretory response (Mason *et al.* 1991; Flezar and Heisler, 1993; Middleton *et al.* 1993; Wilson *et al.* 1994, 1995; Martin and Shuttleworth, 1995). It was therefore proposed that these receptors may allow ATP released from periglandular nerves (see Burnstock, 1990) to contribute to the neural control of epithelial transport processes (Ko *et al.* 1994*a*; Martin and Shuttleworth, 1995; Wilson *et al.* 1995). The present study, however, showed that these receptors were essentially confined to the apical aspect of the cell layer, and there is a growing body of evidence to suggest that this location is typical (Wong, 1988; Mason *et al.* 1991; Xu *et al.* 1996). It is, therefore, now clear that P_{2U} receptors cannot be activated by ATP released from periglandular nerves, and their role in epithelial physiology is enigmatic. It has, however, been suggested that a protein found in the apical membranes of vertebrate epithelia may allow anionic forms of ATP to leave the cell during stimulation with cyclic-AMP-dependent agonists (Reisin *et al.* 1994). Furthermore, it has also been proposed that the ATP leaving the cell in this manner could activate apical P_{2U} receptors. Responses to cyclic-AMP-dependent agonists would, therefore, normally include events due to the autocrine stimulation of P_{2U} receptors (Schwiebert *et al.* 1995).

Equine sweat glands are exocrine organs in which secretory activity is primarily controlled by cyclic-AMP-dependent processes (Snow, 1977; Johnson and Creed, 1982; Bijman and Quinton, 1984*a*,*b*). The present demonstration of apical P_{2U} receptors in a cell line derived from these organs raises the possibility that such receptors may be present in intact glands. It is therefore possible that this novel autocrine control mechanism proposed by Schwiebert *et al.* (1995) may contribute to the control of secretion in these glands. If P_{2U} receptors are activated as part of the normal response to adrenaline, then their inhibitory effect on cyclic AMP formation may lead to a progressive loss of sensitivity to this hormone during prolonged stimulation. Such an autocrine inhibitory pathway could be an important part of the mechanism by which secretion is controlled.

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