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RESEARCH PAPER

Adenosine-evoked Na^+ transport in human airway epithelial cells

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Background and purpose: Absorptive epithelia express apical receptors that allow nucleotides to inhibit Na^+ transport but ATP unexpectedly stimulated this process in an absorptive cell line derived from human bronchiolar epithelium (H441 cells) whilst UTP consistently caused inhibition. We have therefore examined the pharmacological basis of this anomalous effect of ATP.
Experimental approach: H441 cells were grown on membranes and the short circuit current (I_{SC}) measured in Ussing chambers. In some experiments, $[Ca^{2+}]_i$ was measured fluorimetrically using Fura -2. mRNAs for adenosine receptors were determined by the polymerase chain reaction (PCR).
Key results: Cross desensitization experiments showed that the inhibitory response to UTP was abolished by prior exposure to ATP whilst the stimulatory response to ATP persisted in UTP-pre-stimulated cells. Apical adenosine evoked an increase in I_{SC} and this response resembled the stimulatory component of the response to ATP, and could be mimicked by adenosine receptor agonists. Pre-stimulation with adenosine abolished the stimulatory component of the response to ATP. mRNA encoding A_1, A_{2A} and A_{2B} receptor subtypes, but not the A_3 subtype, was detected in H441 cells and adenosine receptor antagonists could abolish the ATP-evoked stimulation of Na^+ absorption.
Conclusions and implications: The ATP-induced stimulation of Na^+ absorption seems to be mediated via $A_{2A/B}$ receptors activated by adenosine produced from the extracellular hydrolysis of ATP. The present data thus provide the first description of adenosine-evoked Na^+ transport in airway epithelial cells and reveal a previously undocumented aspect of the control of this physiologically important ion transport process.
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Keywords: Airway epithelia; epithelial Na^+ transport; Ussing chamber; adenosine receptors; $P2Y_2$ receptor

Abbreviations: A_1, A_2 and A_3 receptor, types 1, 2 and 3 adenosine receptor; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CGS-21680, 2-*p*-(2-carboxyethyl)-phenethylamino-5′-*N*-ethylcarboxamidoadenosine; DPCPX, 1,3-dipropyl-8-cyclopentyl-xanthine; EDTA, ethylenediaminetetraacetic acid; F_{340}, intensity of Fura-2 fluorescence signal evoked by excitation at 340 nM; F_{380}, intensity of Fura-2 fluorescence signal evoked by excitation at 380 nM; FBS, fetal bovine serum; IB-MECA, N^6-(3-iodobenzyl)adenosine-5-*N*-methyluronamide; I_{SC}, short-circuit current; MRS1706, *N*-(4-acetylphenyl)-2-[4-(2,3,6,7-tetahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide; PCR, polymerase chain reaction; PLC, phospholipase C; PKA, protein kinase A; NCS, newborn calf serum; r.u., ratio units; R_t, transepithelial resistance; RT, reverse transcriptase; s.e.m., standard error of the mean; SPA, N^6-(*p*-sulphophenyl)adenosine; UTP, uridine triphosphate; V_t, transepithelial voltage; ZM 241385, 4-(2-[7-amino-2-92-furyl)[1,2,4]triazolo[2,3-a][1.3.5]triazin-5-ylamino]ethyl)phenol

Introduction

Virtually all polarized epithelia express apical receptors that allow nucleotides in the luminal fluid to control the transport of water and salts (see, e.g. Wong, 1988; Mason *et al*., 1991; Wilson *et al*., 1996; Ko *et al*., 1997; Ramminger *et al*., 1999; Leipziger, 2003). One of the most frequently documented such receptors is the $P2Y_2$ receptor, which couples to phospholipase C/inositol-trisphosphate- (PLC/IP_3) (see Berridge, 1993) and thus allows adenosine triphosphate (ATP) and uridine triphosphate (UTP) to increase intracellular free Ca^{2+} ($[Ca^{2+}]_i$) (Nicholas *et al*., 1996). Studies of several different absorptive epithelia have shown that activation of these receptors leads to inhibition of Na^+

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transport (see e.g. Inglis *et al*., 1999, 2000; Ramminger *et al*., 1999; Cuffe *et al*., 2000; Kunzelmann and Mall, 2003; Kunzelmann *et al*., 2005) and, in the airways, these receptors appear to form part of a mechanism that allows autocrine control over the depth of airway surface liquid (Lazarowski *et al*., 2004). These observations suggest that $P2Y_2$ receptor agonists might provide a means of treating the abnormally high rate of Na^+ transport seen in lungs of patients with cystic fibrosis (CF), a lethal genetic disease (Yerxa *et al*., 2002; Leipziger, 2003; Kunzelmann and Mall, 2003). This prompted us to explore the effects of ATP and UTP upon Na^+ transport and $[Ca^{2+}]_i$ in a Na^+ absorbing cell line derived from the human bronchiolar epithelium (H441, see e.g. Sayegh *et al*., 1999; Clunes *et al*., 2004; Ramminger *et al*., 2004). Although our data show that UTP inhibits Na^+ absorption, ATP caused an unexpected stimulation of this process and, as $P2Y_2$ receptors are equally sensitive to these nucleotides (Nicholas *et al*., 1996), an additional receptor must underlie this effect of ATP. The aim of the present study was therefore to establish the pharmacological basis of this unexpected effect of ATP.

Materials and methods

Cell culture

Standard techniques were used to maintain stocks of H441 cells in Rosewell Park Memorial Institute 1640 medium supplemented with fetal bovine serum (FBS, 8.5%), newborn calf serum (NCS, 8.5%), glutamine (2 mM), insulin ($5\,\mu g\,ml^{-1}$), transferrin ($5\,\mu g\,ml^{-1}$), selenium ($5\,ng\,ml^{-1}$) and an antibiotic/antimycotic mixture (Sigma Chemical Co., Poole, Dorset, UK). For experiments, cells removed from the culture flasks using trypsin/ethylene diaminetetraacetic acid, were resuspended in standard culture medium and plated ($\sim 10^6\,cells\,cm^{-2}$) onto Costar Snapwells (Corning BV, Schipol-Rijk, The Netherlands). For experiments in which short-circuit current (I_{SC}) and $[Ca^{2+}]_i$ were measured simultaneously, these membranes were cut into small pieces that were glued to Perspex disks with 1 mm holes drilled through them to form small wells into which the cells were seeded (Ko *et al*., 1999). After $\sim$24 h, all cultures were gently washed to remove non-adherent cells and incubated (4–6 days) in medium identical to that described above, except that (i) FBS and NCS were replaced with FBS (8.5%) that had been dialysed against 150 mM NaCl to remove hormones and growth factors, and (ii) it contained 0.2 μM dexamethasone, a synthetic glucocorticoid that induces a Na^+ absorbing phenotype in these cells (Sayegh *et al*., 1999; Clunes *et al*., 2004; Ramminger *et al*., 2004).

Standard electrometric measurements

Costar Snapwell membranes bearing confluent cells (see above) were mounted in Ussing chambers and bathed with physiological saline (composition 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 (pH 7.3–7.5), when bubbled with 5% CO_2) that was maintained at 37°C and continuously circulated using gas lifts. The transepithelial potential (V_t) was initially monitored under open-circuit conditions and, once this stabilized (20–30 min), V_t was clamped at 0 mV (DVC 1000 voltage/current clamp, World Precision Instruments, Stevenage, Herts, UK), whereas the current needed to maintain this potential (I_{SC}) was monitored and recorded (4 Hz) using a PowerLab interface (AD Instruments, Hastings, East Sussex, UK). Transepithelial resistance (R_t) was determined from the expression $R_t = V_t/I_{SC}$. In some experiments, physiological saline was continually pumped through the apical and basolateral chambers so that applied drugs could subsequently be washed from the bath.

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

Confluent cells (see above) were loaded with Fura-2 by incubation ($\sim$40 min, 37°C) in medium containing the acetoxymethyl ester form (3 μM) of this Ca^{2+}-sensitive fluorescent dye together with pluronic F127 (1.8 μM), a non-ionic detergent, and probenecid (2.5 mM), an inhibitor of organic cation extrusion systems. Confluent H441 cells accumulate little dye under standard conditions and so inclusion of these compounds was necessary for adequate dye loading. The Fura-2-loaded cells were mounted in a miniature Ussing chamber (Lazarowski *et al*., 1997; Ko *et al*., 1999) where the basolateral and apical sides of the cell layer were independently superfused ($\sim 3\,ml\,min^{-1}$, 37°C) with physiological saline (see above). Solenoid operated valves allowed these solutions to be independently and rapidly switched and, immediately before entering the chambers, the solutions passed through thermostatically controlled heaters to ensure that experiments were carried out at 37°C. The chamber was mounted on the stage of a Nikon inverted microscope equipped with extra long working distance fluorescence optics (Nikon CFI Plan Fluor ELWD, 0.6 numerical aperture) and a Cairn Research (Faversham, Kent, UK) Optoscan UV light source so that the cells could be alternatively illuminated at 340 and 380 nM. The intensity of fluorescence (510 nM) evoked by illumination at these two wavelengths (intensity of Fura-2 fluorescence signal evoked by excitation at 340 nM (F_{340}) and intensity of Fura-2 fluorescence signal evoked by excitation at 380 nM (F_{380}), respectively) was monitored and recorded (4 Hz) in parallel with I_{SC}, which was recorded using a Physiologic Instruments (San Diego, CA, USA) VCC600 voltage clamp. A full account of this method is published elsewhere (Ko *et al*., 1999).

Acute changes in the ratio F_{340}/F_{380} are often used as an indicator of $[Ca^{2+}]_i$, but such experiments are usually undertaken using cells plated onto glass coverslips, whereas confluent cells on membranes take up less dye than single cells and substantial amounts of light are scattered by the culture membrane, which substantially increases background fluorescence. Under these conditions, the fraction of F_{340} and F_{380} owing to background can be substantial and, as Fura-2 fluorescence will decline as the dye is bleached and/or extruded from the cell (Grynkiewicz *et al*., 1985), the relative contribution of this background will change throughout the experiments. To ensure that this did not confound analysis of the present data, at the end of each experiment the cells were exposed to thapsigargin (1 μM,

bilateral), a substance that evokes a substantial rise in $[Ca^{2+}]_i$ (see Thastrup *et al*., 1990). Once this rise was fully established, the cells were exposed to *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulphonic acid-buffered saline containing 10 mM $MnCl_2$. As the thapsigargin-activated Ca^{2+}-influx pathway is Mn^{2+}-permeable (e.g. Jacob, 1990), and as Mn^{2+} rapidly quenches Fura-2 fluorescence, this causes a rapid fall in F_{340} and F_{380}. The Mn^{2+}-resistant component of each signal was assumed to indicate the cation-insensitive background fluorescence present throughout the preceding experiment. Files encoding the raw data were transferred to a computer spreadsheet (Microsoft *Excel*) and the appropriate background values subtracted before the ratio F_{340}/F_{380} was calculated.

Experimental design and data analysis

All drug applications were timed carefully so that individual records could be aligned and pooled to give overall 'mean' traces that are shown as mean ± s.e.m.; these manipulations were undertaken using the standard features of Microsoft *Excel*. As the spontaneous I_{SC} recorded from different cell preparations could vary over a substantial range (5–50 $\mu A\,cm^{-2}$), experiments were undertaken using strictly paired protocols in which the control and experimental cells were age matched and were at identical passage. The significance of differences between mean values was thus assessed using Student's paired *t*-test or one-way analysis of variance as appropriate. Experimentally induced changes in I_{SC} and F_{340}/F_{380} were determined, for individual experiments, by subtracting the values measured in unstimulated cells from the values measured during stimulation and are also presented as mean ± standard error of the mean (s.e.m.). Desensitization experiments were undertaken using a standard protocol in which the cells were exposed to the initial, desensitizing agonist for 10 min, and washed with physiological saline for 10 min before being stimulated a second time.

Isolation/analysis of RNA

RNA was isolated from cells ($\sim 3 \times 10^6$) grown on Costar Transwell membranes (Corning BV, Schipol-Rijk, The Netherlands) using Trizol reagent (Gibco BRL, Paisley, UK) and aliquots (5 μg) of this RNA fractionated on formaldehyde/agarose/ethidium bromide gels and it was examined under UV light to ensure that significant degradation had not occurred. Separate aliquots (2 μg) were then transcribed into cDNA using M-murine leukemia virus reverse transcriptase (RT) (200 U μl^{-1}) and excess (10 mM) dATP, dCTP, dGTP and dTTP. Aliquots of the resultant cDNA corresponding to 1 μg of RNA then served as templates in the polymerase chain reaction (PCR) using the primers detailed in Table 1. All such reactions took place in 50 μl of PCR buffer containing 1.5 mM Mg^{2+}, 1 μM of the appropriate sense and antisense primers, and excess (200 μM) dATP, dCTP, dGTP and dTTP. Samples were first denatured at 95°C for 3 min and the PCR reaction then allowed to proceed through 40 denaturing (1 min at 95°C), annealing (1.5 min at the temperatures given in Table 1) and polymerization (2 min at 72°C) cycles followed by a final polymerization period (10 min) at 72°C. The resultant products were fractionated on agarose/ethidium bromide gels and their sizes determined by reference to known standards. Products were isolated from the gels and sequenced (ABI 3100 Genetic Analyser; Ninewells Hospital and Medical School DNA Analysis Facility, University of Dundee) to verify their origin. PCR reactions were also undertaken using (i) aliquots of extracted RNA that had not been exposed to RT, but which had otherwise been treated identically and (ii) aliquots of water containing neither DNA nor RNA. Moreover, to confirm that the failure to generate a particular product was not owing to a failure the RT-PCR process itself, all assays were run in parallel with control reactions using actin-specific primers.

Results

Verification of methodology

The mean values of R_t, I_{SC} and V_t for cells mounted in standard Ussing chambers were $257.8 \pm 5.7\,\Omega cm^2$, $44.6 \pm 3.3\,\mu A\,cm^{-2}$ and 11.7 ± 1.1 mV, respectively ($n > 60$), and these values differed from the equivalent data derived from Fura-2-loaded cells mounted in the miniature Ussing chamber (R_t: $387.5 \pm 14.8\,\Omega cm^2$; I_{SC}: $10.5 \pm 0.5\,\mu A\,cm^{-2}$; V_t: 4.5 ± 0.3 mV, $n > 60$). However, both data sets lie within the range of values previously reported for these cells (see also Sayegh *et al*., 1999; Lazrak and Matalon, 2003; Ramminger *et al*., 2004) and our experience is that there can be day-to-day variations in the magnitudes of these parameters. We therefore directly explored the effects of Fura-2 loading and of exposure to the solvent vehicle (0.1% dimethyl sulphoxide (DMSO)) using a strictly paired protocol ($n = 6$). These manoeuvres did not affect R_t (control: $191.7 \pm 12.0\,\Omega cm^2$, DMSO: $187.8 \pm 23.8\,\Omega cm^2$; Fura-2: $227.8 \pm 21.9\,\Omega cm^2$) or I_{SC}

Table 1 Primers used in PCR reactions

Target sequence	*Forward primer*	*Reverse primer*	*Size* (kb)	T_A (°C)
$P2Y_2$ (NM002564)	TCAATGGCACCTGGGATG	ATGTCCTTAGTGTTCTCGGCT	1.1	58
A_1 (NM000674)	ATGCCGCCCTCCATCTCA	CTAGTCATCAGGCCTCTCT	0.98	60
A_{2A} (NM000675)	ATCATGGGCTCCTCGGTGTA	GGACACTCCTGCTCCA	1.2	62
A_{2B} (NM000676)	ATGCTGCTGGAGACACAGGA	ACACCGAGAGCAGGTGTAC	0.98	62
A_3 (NM000677)	ATGCCCAACAACAGCACT	TCAGAATTCTTCTCAAGCT	0.96	54

Abbreviations: PCR, polymerase chain reaction; T_A, annealing temperature.
The primers sequences were designed to generate PCR products specific to the listed receptor subtypes by reference to the gene sequences referenced by accession numbers given in parentheses. The table also lists the predicted sizes of the appropriate PCR products and T_A.

(control: $28.2 \pm 2.9\,\mu A\,cm^{-2}$; DMSO: $29.8 \pm 4.0\,\mu A\,cm^{-2}$; Fura-2: $32.8 \pm 1.8\,\mu A\,cm^{-2}$), but Fura-2 caused a small rise ($P<0.05$) in V_t (control: 5.3 ± 0.4 mV, DMSO: 5.3 ± 0.6 mV; Fura-2: 7.4 ± 0.7 mV). Measurements made after 10 μM amiloride had been added to the apical bath revealed no effect upon the fraction of the spontaneous I_{SC} that was inhibited by this Na^+ channel antagonist (control: $90.4 \pm 3.2\%$, DMSO: $86.3 \pm 6.3\%$, Fura-2: $87.0 \pm 5.0\%$). Several of the compounds used in this study were insoluble in water and required the use of DMSO as a solvent vehicle. Preliminary experiments showed that cells tolerated 0.1% DMSO without detrimental effect, whereas higher concentrations led to a fall in I_{SC}. The total DMSO concentration therefore did not exceed 0.1% (vol/vol) in any experiment.

Effects of ATP and UTP upon I_{SC}

Figure 1 shows the effects of adding 100 μM ATP (Figure 1a) or UTP (Figure 1b) to the apical solution. Initially, both nucleotides caused rapid increases in I_{SC}, but these responses were transient and I_{SC} had thus returned to its basal value within ~3 min. In ATP-stimulated cells, this initial transient was followed by a second rise in I_{SC} that developed more slowly, taking ~10 min to reach a plateau value that was then maintained for at least 40 min (Figure 1a). In contrast, the initial response to UTP was followed by a progressive fall in I_{SC} so that the current recorded after ~5 min lay below the value measured at the onset of the experiments (Figure 1b). This inhibitory response was not maintained as the I_{SC} slowly returned to its initial value over ~10 min (Figure 1b). Approximately 30 min after the addition of the first nucleotide, a dose (100 μM) of the other nucleotide was applied. Thus, UTP was added to ATP-stimulated cells (Figure 1a) and ATP to UTP-stimulated cells (Figure 1b). Figure 1a shows that UTP had no discernible effect upon I_{SC} across ATP-stimulated cells. Figure 1b, on the other hand, shows that prestimulation with UTP abolished the initial component of the response to ATP, but did not affect the second, slowly developing increase in I_{SC}. Figure 1 also shows that apical amiloride (10 μM) reduced the recorded current to ~15% of control and abolished the nucleotide-evoked changes in I_{SC} (Figure 1a and b).

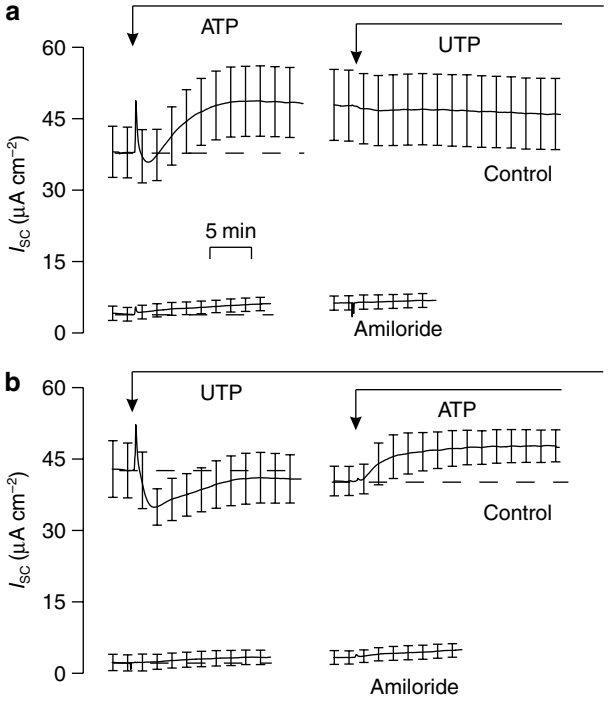

Figure 1 Effects of ATP and UTP on ion transport (I_{SC}) measured across cells mounted in standard Ussing chambers either under control conditions ($n=4$) or in the presence of 10 μM apical amiloride ($n=3$). (**a**) Changes in I_{SC} induced by adding ATP followed by UTP (both 100 μM) to the apical solution. (**b**) Effect of adding UTP followed by ATP (both 100 μM). Data are mean ± s.e.m.

Effects of ATP and UTP upon I_{SC} *and* F_{340}/F_{380}

Figure 2a shows that the ATP-evoked changes in I_{SC} described above are accompanied by a rise in F_{340}/F_{380} that consists of an initial transient followed by a decline to a second more slowly developing increase. Similarly, the UTP-evoked changes in I_{SC} are also accompanied by an increase in $[Ca^{2+}]_i$ and, although these data suggest that this response is smaller than the corresponding response to ATP, this was not a consistent finding (see e.g. Figure 4). Neither nucleotide (ATP, $n=6$; UTP, $n=4$; both 100 μM) had a discernible effect upon I_{SC} or F_{340}/F_{380} when added to the basolateral solution. In subsequent experiments, cells were exposed to two pulses of apical ATP (100 μM) separated by a 10 min recovery period (see Materials and methods). The first such application evoked increases in I_{SC} and F_{340}/F_{380} similar to those described above and, while the second stimulus did evoke discernible increases in both signals, analysis showed that the initial, transient component of the electrometric response and the associated rise in $[Ca^{2+}]_i$ were essentially abolished (Figure 3a and b). Moreover, while a slowly developing increase in I_{SC} was evident, this was smaller than control (Figure 3a). Similarly, experiments in which cells were repeatedly stimulated with UTP (100 μM) showed that both components of the response to the second application of this nucleotide were greatly reduced (Figure 3c and d).

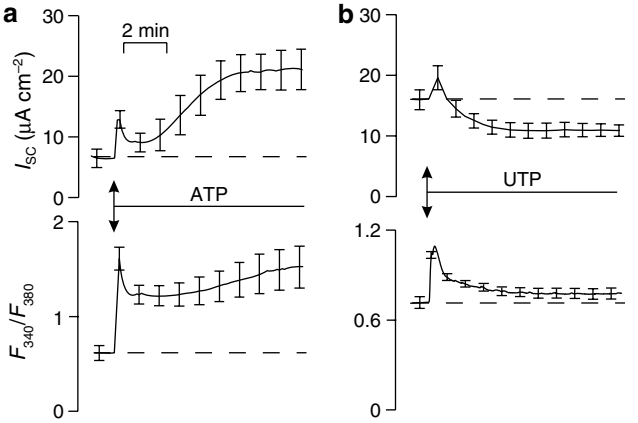

Figure 2 Effects of ATP and UTP on ion transport (I_{SC}) and Fura-2 fluorescence ratio (F_{340}/F_{380}) measured simultaneously. (**a**) Changes in the two parameters evoked by 100 μM ATP ($n=33$) (**b**) Effect of 100 μM UTP ($n=22$). Both signals are presented as mean ± s.e.m.

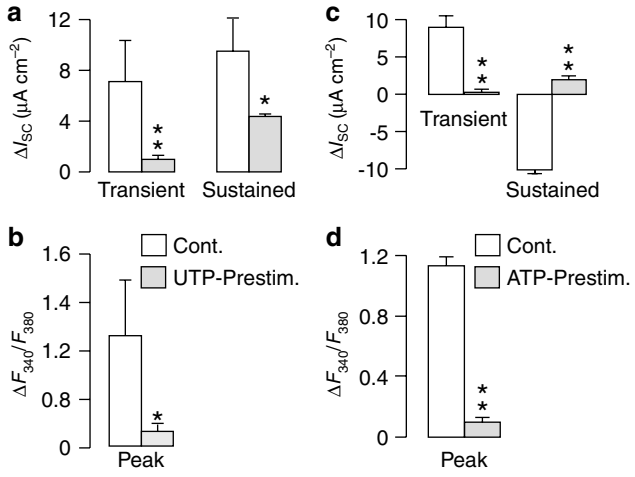

Figure 3 Effect of consecutive doses (100 μM apical) of ATP (**a** and **b**; $n=5$) and UTP (**c** and **d**; $n=5$) upon I_{SC} and F_{340}/F_{380} measured simultaneously. Both the initial peak increase in I_{SC} ('Transient') and the increase after 10 min exposure to the nucleotide ('Sustained') are presented as mean$\pm$s.e.m. (**a** and **c**). The peak increases in Ca^{2+} ($\Delta F_{340}/F_{380}$) evoked by the first and second applications of nucleotide were also quantified ('Peak'; mean$\pm$s.e.m.; **b** and **d**) Asterisks denote statistically significant differences between the responses evoked by the first and second applications of nucleotide (*$P<0.05$, **$P<0.002$).

The responses to ATP and UTP are thus subject to autologous desensitization and subsequent studies explored the extent to which ATP and UTP could cause cross-desensitization. Figure 4a thus shows that prior stimulation with ATP essentially abolishes the UTP-evoked changes in I_{SC} (Figure 4e) and F_{340}/F_{380}. Figure 4b, on the other hand, shows that prestimulation with UTP abolishes the initial transient component of the ATP-evoked rise in I_{SC} (Figure 4c), attenuates the increase in F_{340}/F_{380} (Figure 4d), but has no statistically significant effect upon the sustained increase in I_{SC} (Figure 4c). A receptor population that does not desensitize during exposure to UTP thus underlies the sustained component of the response to ATP.

Effects of adenosine receptor antagonists upon the response to ATP
Figure 5 shows electrometric responses to ATP in control cells (a) and in cells that had been pretreated (20 min) with 4-(2-[7-amino-2-92-furyl)[1,2,4]triazolo[2,3-a][1.3.5]triazin-5-ylamino]ethyl)phenol (ZM 241385) (b) *N*-(4-acetylphenyl)-2-[4-(2,3,6,7-tetahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)-phenoxy]-acetamide (MRS 1706) (c) or 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (d; all 1 μM), compounds that all act as adenosine receptor antagonists. These substances did not affect basal I_{SC} or the initial, transient component of the response to ATP (control: $6.3\pm1.5\,\mu A\,cm^{-2}$; ZM 241385: $5.6\pm1.7\,\mu A\,cm^{-2}$; MRS 1706: $2.7\pm0.7\,\mu A\,cm^{-2}$; DPCPX: $4.7\pm1.5\,\mu A\,cm^{-2}$), but reduced the second, more sustained component of this response. The effect of each antagonist was quantified by measuring the current recorded after 15 min exposure to ATP. This normally exceeded the basal I_{SC} by $14.5\pm1.9\,\mu A\,cm^{-2}$ and all of the tested antagonists significantly ($P<0.05$) inhibited this current (ZM 241385: $3.3\pm1.2\,\mu A\,cm^{-2}$; MRS 1706: $1.1\pm1.1\,\mu A\,cm^{-2}$; DPCPX: $4.4\pm1.3\,\mu A\,cm^{-2}$). There were no statistically significant differences amongs the data derived from the antagonist-treated cells.

Effects of ATP-γ-S
Initial experiments ($n=4$) showed that ATP-γ-S caused a transient increase in I_{SC} ($\Delta I_{SC}=6.6\pm1.8\,\mu A\,cm^{-2}$) that was followed by a fall to a value below baseline that was reached after $\sim$5 min ($\Delta I_{SC}=6.3\pm2.7\,\mu A\,cm^{-2}$), but gave way to a slowly developing stimulation so that the I_{SC} recorded after 15 min exceeded the basal I_{SC} by $8.4\pm1.0\,\mu A\,cm^{-2}$. This response was thus very similar to that evoked by ATP itself (Figure 1). However, a different picture emerged from experiments undertaken using a second sample of ATP-γ-S, obtained from the same supplier but with a different batch number. The initial phases of this response were as described above (Figure 6a) but, in these experiments, the I_{SC} recorded after 15 min exceeded the basal I_{SC} by only $4.2\pm0.4\,\mu A\,cm^{-2}$ and was thus smaller ($P<0.05$) than that described above. ZM 241385 (1 μM, 20 min preincubation) had no effect on the initial phases of the response to ATP-γ-S, but, under these conditions, the initial transient was followed by a sustained inhibitory phase and I_{SC} remained below its basal level for the remainder of the experiment (Figure 6b). The purity of the second batch of ATP-γ-S used was assessed by high-performance liquid chromatography (HPLC) (see Lazarowski *et al*., 2004). This analysis showed that a solution with a nominal ATP-γ-S concentration of 100 μM contained a substantial amount of adenosine diphosphate (ADP) ($\sim$15 μM) and low levels of adenosine ($\sim$0.01 μM), adenosine monophosphate (AMP) ($\sim$0.1 μM) and ATP ($\sim$0.3 μM). We did not analyse the first batch of ATP-γ-S used as we were unable to obtain a further sample.

Effects of adenosine
Apical adenosine (200 μM) caused a slowly developing and sustained rise in I_{SC} that became apparent after 2–3 min and reached a plateaux value after $\sim$8 min (Figures 7 and 10a). Examination of the simultaneously recorded values of F_{340}/F_{380} showed that parameter rose slowly from 0.54 ± 0.06 to 0.72 ± 0.17 ($P<0.05$), demonstrating that this increase in I_{SC} was accompanied by a modest increase in $[Ca^{2+}]_i$. However, this effect was not observed in all experiments and adenosine did not evoke rapid increases in F_{340}/F_{380} like those seen during stimulation with ATP or UTP. Adenosine also increased I_{SC} when added to the basolateral solution (basal I_{SC}: $4.5\pm0.4\,\mu A\,cm^{-2}$; adenosine-stimulated: $13.7\pm2.4\,\mu A\,cm^{-2}$, $n=4$, $P<0.01$) and, qualitatively, this response was similar to that evoked by apical adenosine. Although F_{340}/F_{380} appeared to rise slightly during stimulation with basolateral adenosine (basal F_{340}/F_{380}: 0.38 ± 0.05; adenosine stimulated F_{340}/F_{380}: 0.55 ± 0.009), this did not reach statistical significance. Experiments in which cells were successively exposed to two pulses of apical adenosine showed that the both stimuli caused significant ($P<0.05$ for both) increases in I_{SC}, although the first response (ΔI_{SC}; 6.3 ± 1.4 $17.0\pm5.4\,\mu A\,cm^{-2}$) was larger ($P<0.05$) than the

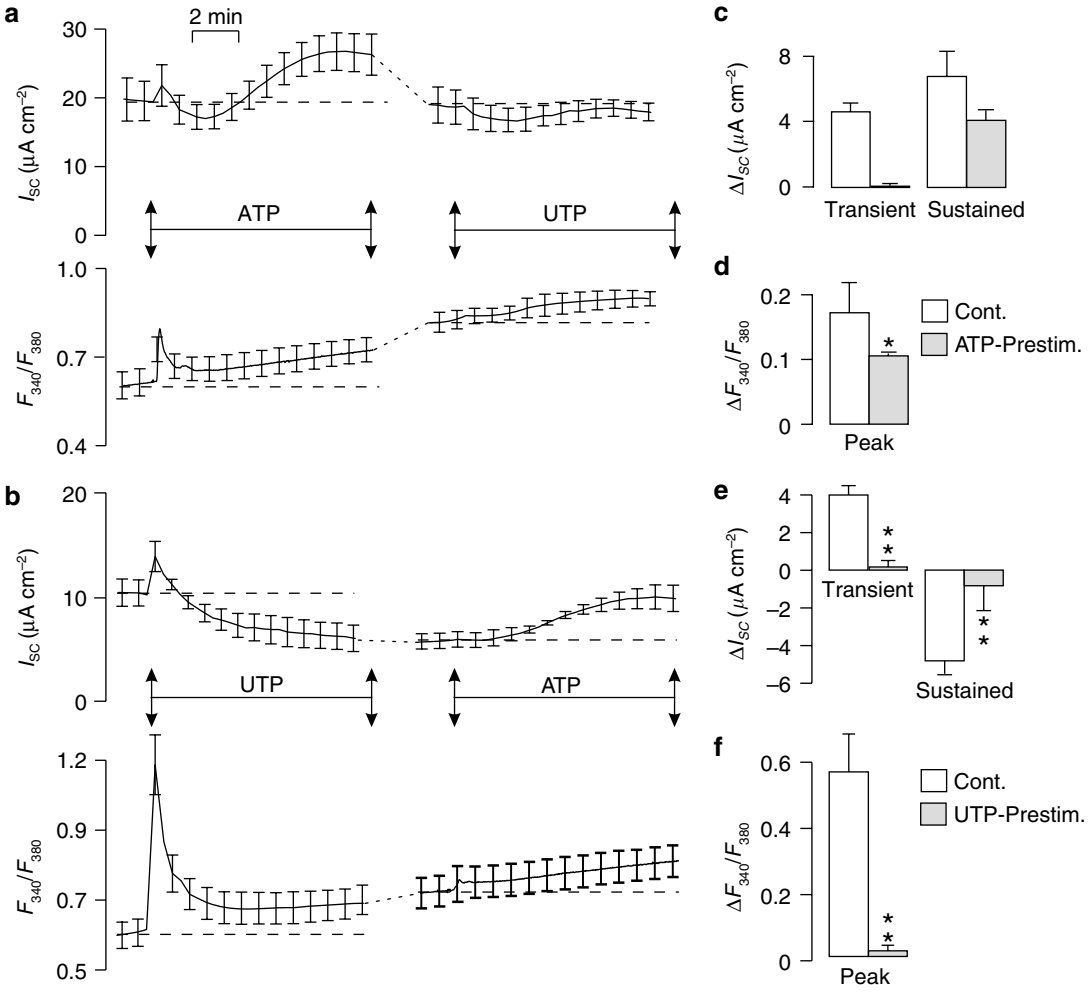

Figure 4 Effect of ATP followed by UTP (**a**, **c** and **d**) and UTP followed by ATP (**b**, **e** and **f**) on I_{SC} and F_{340}/F_{380} measured simultaneously. (**a**) and (**b**) show the time courses of the cross-desensitization experiments. The dashed lines represent a 10 min wash with control saline. Both the initial peak increase in I_{SC} ('Transient') and the increase after 10 min exposure to the nucleotide ('Sustained') are shown (**c** and **e**). The peak increases in Ca^{2+} ($\Delta F_{340}/F_{380}$) evoked by the first and second applications of nucleotide were also quantified ('Peak'; **d** and **f**). All data are mean ± s.e.m. ($n=5$) and asterisks denote statistically significant differences between the data derived from control and prestimulated cells (*$P<0.05$, **$P<0.02$).

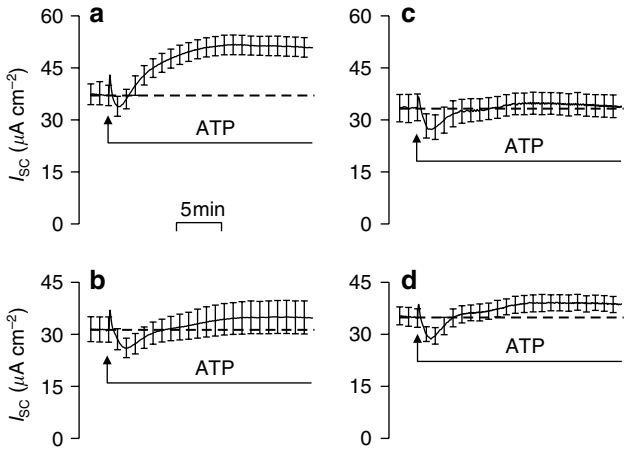

Figure 5 Effect on I_{SC} (mean ± s.e.m.) of apical ATP (100 μM) under control conditions (**a**; $n=7$) and in cells that had been preincubated (20 min) in ZM 241385 (**b**; 1 μM, $n=4$), MRS 1706 (**c**; 1 μM, $n=4$), or DPCPX (**d**; 1 μM, $n=4$).

second (ΔI_{SC}; 2.4 ± 0.9 μA cm^{-2}), indicating that the receptors underlying this response are subject to autologous desensitization. Although F_{340}/F_{380} seemed to increase during both adenosine pulses, neither change reached statistical significance.

Desensitizing interactions between adenosine and ATP

Figure 8 shows ATP-evoked changes in I_{SC} and F_{340}/F_{380} measured in control and adenosine-prestimulated (100 μM, apical) cells. The control data (Figure 8a) confirm the ATP-evoked changes in I_{SC} and $[Ca^{2+}]_i$ described above and, although ATP also increased I_{SC} in the adenosine-prestimulated cells (Figure 8b), the initial part of this response was normal, whereas the second, slowly developing component did not occur (Figure 8c). Prestimulation with adenosine had no effect upon the ATP-evoked increase in $[Ca^{2+}]_i$ (Figure 8b and d). Further experiments showed that prestimulation with ATP reduced, but did not abolish the adenosine-evoked

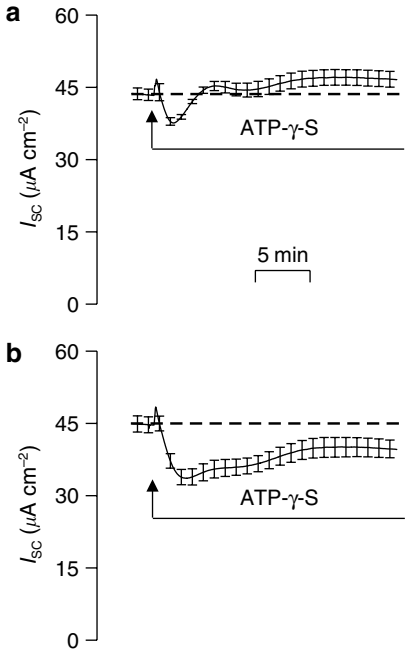

Figure 6 Effect of apical ATPγS (100 μM) on I_{SC}. (**a**) Effect under control conditions and (**b**) Effect on cells that had been preincubated (20 min) in ZM 241385 (1 μM). Data are mean±s.e.m., $n=4$.

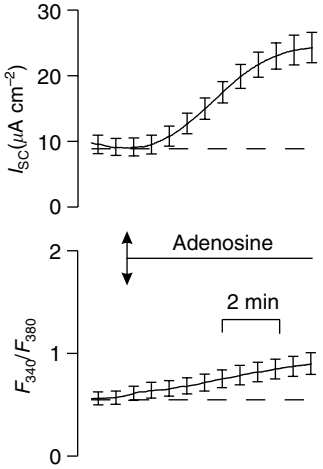

Figure 7 Effects of apical adenosine (100 μM) upon I_{SC} (upper panel) and F_{340}/F_{380} (lower panel). Data are mean±s.e.m. ($n=5$).

increase in I_{SC} (control ΔI_{SC}: 15.2±5.3 μA cm^{-2}; ATP-prestimulated ΔI_{SC}: 8.8±0.9 μA cm^{-2}, $n=5$; $P<0.01$).

Ionic basis of the response to adenosine

Studies ($n=4$) of bi-laterally superfused epithelial cell layers (see Materials and methods) showed that apical amiloride (10 μM) rapidly reduced the spontaneous I_{SC} from 26.9±6.1 μA cm^{-2} to 4.4±1.3 μA cm^{-2} ($P<0.001$) and they established that this inhibitory effect was reversible, and thus the I_{SC} recorded ~10 min after amiloride had been washed from the bath (20.9±4.4 μA cm^{-2}) did not differ from that measured at the onset of the experiments. Subsequent application of apical adenosine (200 μM) evoked a slowly developing rise in I_{SC} and the I_{SC} measured after ~10 min (36.0±4.9 7 μA cm^{-2}) exceeded ($P<0.05$) the basal value. Subsequent addition of apical amiloride (10 μM) reduced the I_{SC} recorded from these cells to 6.1±1.4 μA cm^{-2} and analysis of these data showed that stimulation with adenosine had caused a substantial increase in amiloride-sensitive I_{SC} (control: 22.5±5.7 μA cm^{-2}; adenosine stimulated 29.9±4.3 7 μA cm^{-2}; $P<0.05$). As such control over amiloride-sensitive I_{SC} can reflect changes in apical Na^+ conductance (G_{Na+}) (see e.g. Morris and Schafer, 2002; Ramminger *et al.*, 2004), we explored the effects of adenosine upon this parameter by measuring the apical membrane currents in basolaterally permeabilized cells exposed to an inwardly directed Na^+ gradient (see Materials and methods). Initial measurements of intact cells ($n=6$) showed that apical adenosine (200 μM) increased I_{SC} from 37.6±3.5 to 57.5±5.3 μA cm^{-2} ($P<0.0005$), and measurements made after permeabilization showed that G_{Na+} was 354.9±41.37 μS cm^{-2}. This value is greater ($P<0.001$) than that measured in unstimulated cells (213.5±36.5 μS cm^{-2}) at identical passage that generated 36.5±2.7 μA cm^{-2} of spontaneous I_{SC}.

Pharmacological basis of the response to adenosine

To characterize the receptors underlying the adenosine-evoked increase in I_{SC}, we first attempted to construct cumulative concentration response curves by progressively raising the concentration of adenosine in the apical solution. However, although the cells consistently responded to adenosine, this approach was not fruitful as the responses to low concentrations developed slowly and it was very difficult to discern clear plateaux values as the concentration was increased. We therefore adopted an alternative strategy in which individual monolayers were exposed to only a single agonist concentration. Although this allows us to explore the pharmacological properties of the adenosine-sensitive receptors, variability between different monolayers, both in the basal I_{SC} and in the magnitude of response to a given concentration of adenosine, imposed an unavoidable limitation upon the quality of the data that could be obtained in this way. The data in Figure 9, which were obtained in this way, thus show that the response to adenosine is concentration dependent. The adenosine-evoked increases in I_{SC} measured in these experiments have been plotted against the concentration used in Figure 9b and show that the EC_{50} for this compound is 1.6±0.7 μM. Figure 9c shows the increases in I_{SC} evoked by three adenosine analogues N^6-(*p*-sulphophenyl)adenosine (SPA), 2-*p*-(2-carboxyethyl)-phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS 21680) and N^6-(3-iodobenzyl)adenosine-5-*N*-methyluronamide (IB-MECA); the EC_{50} values for these compounds were 1.5±0.3, 8.4±0.9 and 6.2±1.5 μM, respectively. This analysis was undertaken by assuming that the tested drugs bind to a single site as effectively as adenosine (Figure 9), as the variability in the primary data (see above) meant that this simple model could not be

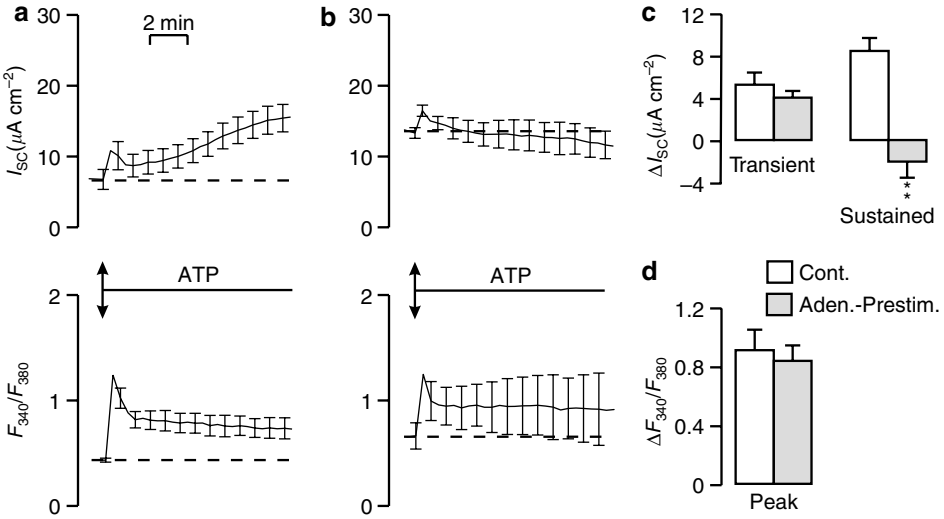

Figure 8 Effect of adenosine on response to ATP. (**a**) Effect of ATP on I_{SC} and F_{340}/F_{380}. (**b**) Effect of ATP on cells that had been stimulated with 100 μM apical adenosine and then washed with control saline for 10 min. (**c**) The initial peak increase in I_{SC} ('Transient') and the increase after 10 min exposure to ATP ('Sustained'). (**d**) The peak increases in Ca^{2+} ($\Delta F_{340}/F_{380}$) evoked by ATP ('Peak'; **d** and **f**). All data mean $\pm$ s.e.m. ($n=5$) and asterisks denote statistically significant differences between control and adenosine prestimulated cells (**$P<0.02$).

distinguished from more complex schemes involving multiple binding sites.

Figure 10a shows that a maximally effective (200 μM) concentration of apical adenosine consistently evoked a sustained increase in I_{SC} essentially identical to that described above. Once this response was established, these cells were exposed to DPCPX, ZM 241385 or MRS 1706 (all 1 μM), and Figure 10b shows the effects of these compounds upon the recorded I_{SC} that was subsequently recorded from these cells. These data have been normalized to the adenosine-evoked increase in I_{SC} measured in each experiment and thus show the changes in I_{SC} that occur during exposure to these adenosine receptor antagonists or solvent vehicle (control). The I_{SC} normally declines by $\sim$20% over the time scale of these experiments and DPCPX (1 μM) had no effect upon this spontaneous fall in I_{SC} (Figure 10b). However, ZM 241385 and MRS 1706 accelerated this decline and analysis of these data confirmed that these compounds significantly reduced the adenosine-stimulated current (Figure 10c). Subsequent experiments indicated that these inhibitory effects were concentration dependent and, although ZM 241385 appeared slightly more potent, we could not discern an unambiguous difference between the effects of these compounds (Figure 10d).

Expression of mRNA encoding $P2Y_2$ and adenosine receptors

RT-PCR-based analyses (see Materials and methods) showed that mRNA transcripts encoding the $P2Y_2$, A_1, A_{2A} and A_{2B} receptors were all expressed by in H441 cells that had been grown to confluence on permeable supports. However, reactions run using primers designed to amplify a sequence characteristic to the A3 receptor subfamily consistently failed to generate any such products, whereas parallel analyses using actin-specific primers confirmed that the assay procedure itself was working normally.

Discussion

Our initial studies confirmed (Sayegh *et al*., 1999; Lazrak and Matalon, 2003; Ramminger *et al*., 2004) that H441 cells spontaneously generate an I_{SC} that is largely ($\sim$85%) due to electrogenic Na^+ absorption and established that loading the cells with Fura-2 had no major effect upon this process. Experiments in which the cells were exposed to ATP or UTP showed that these nucleotides controlled this transport process and increased $[Ca^{2+}]_i$, and the receptors underlying these responses appeared to be confined to the apical membrane. This was not altogether surprising as mRNA encoding the $P2Y_2$ receptor, which is equally sensitive to ATP and UTP, was present and these receptors are found in the apical membranes of many polarized epithelia where they allow luminal nucleotides to increase $[Ca^{2+}]_i$ by activating PLC/IP_3 (Wong, 1988; Brown *et al*., 1991; Mason *et al*., 1991; Nicholas *et al*., 1996; Wilson *et al*., 1996, 1998; Inglis *et al*., 1999; Ramminger *et al*., 1999; Leipziger, 2003; Kunzelmann *et al*., 2005; Wolff *et al*., 2005). The response to UTP consisted of an initial transient stimulation of I_{SC} that was accompanied by a monotonic increase in $[Ca^{2+}]_i$ followed by a slowly developing fall in I_{SC} to a value below control. This resembles that described in other absorptive epithelia and therefore accords with the view that apical $P2Y_2$ receptors allow luminal nucleotides to inhibit Na^+ absorption (see e.g. Inglis *et al*., 1999, 2000; Ramminger *et al*., 1999; Cuffe *et al*., 2000; Kunzelmann and Mall, 2003; Lazarowski *et al*., 2004; Kunzelmann *et al*., 2005). Initially, ATP also evoked a transient rise in I_{SC} resembling that seen during stimulation with UTP and this response was also accompanied by increased $[Ca^{2+}]_i$. However, this initial transient was followed by a slowly developing and sustained stimulation that was in marked contrast to the inhibitory effect of UTP. This discrepancy shows that the electrometric effects of these nucleotides cannot simply be attributed to $P2Y_2$ receptors as

these are equally sensitive to ATP and UTP (see e.g. Nicholas *et al*., 1996).

The anomalous response to ATP

Cross-desensitization experiments showed that the receptors underlying all components of the response to UTP (i.e. the effects on $[Ca^{2+}]_i$ and I_{SC}) were subject to ATP-induced desensitization. However, although the initial components of the response to ATP were similarly abolished by prior exposure to UTP, the sustained increase in I_{SC} persisted in UTP-prestimulated cells, and, under these conditions, this response occurred with no overt change in $[Ca^{2+}]_i$. This implies that this part of the response cannot be attributed to a P2Y receptor as all such receptors couple to PLC/IP_3 and thus cause rapid increases in $[Ca^{2+}]_i$ (Berridge, 1993; Nicholas *et al*., 1996). It was therefore interesting that adenosine receptor antagonists selectively inhibited this part of the response to ATP, suggesting that the stimulation may actually be mediated by adenosine receptors.

Four adenosine receptor subtypes (A_1, A_{2A}, A_{2B} and A_3) have been described (Ralevic and Burnstock, 1998; Fredholm *et al*., 2001) and mRNA encoding the A_1, A_{2A} and A_{2B} receptors, but not the A_3 receptor, could be found in H441 cells, a result that accords with studies of other airway epithelial cell types (Lazarowski *et al*., 1992; Clancy *et al*., 1999; Fredholm *et al*., 2001; Huang *et al*., 2001; Cobb *et al*., 2002, 2003). However, adenosine receptors are characteristically insensitive to ATP (Ralevic and Burnstock, 1998; Fredholm *et al*., 2001) and so it was not immediately apparent how their expression could explain the sustained increase in I_{SC} described here. A possible resolution of this contradiction came from the fact that epithelial cells express ectonucleotidases that allow ATP in contact with the cell surface to be rapidly converted to adenosine, and it is now clear that adenosine formed in this way can activate specific receptors and thus contribute to the control of epithelial Cl^- secretion (see e.g. Huang *et al*., 2001; Lazarowski *et al*., 2004).

This prompted us to explore the effects of ATP-γ-S, a nucleotide that can activate $P2Y_2$ receptors (Nicholas *et al*., 1996), but which is thought to be resistant to such hydrolysis, suggesting that it would evoke a response resembling that seen during stimulation with UTP. However, although the cells consistently responded to ATP-γ-S, our initial data indicated that this nucleotide induced a sustained stimulation of I_{SC} as effectively as ATP. Interestingly, this response was not observed in a second series of experiments undertaken using a different batch of ATP-γ-S (see Materials and methods), although, even in these studies, sustained inhibition was not seen unless the cells were pretreated with an adenosine receptor antagonist. Although

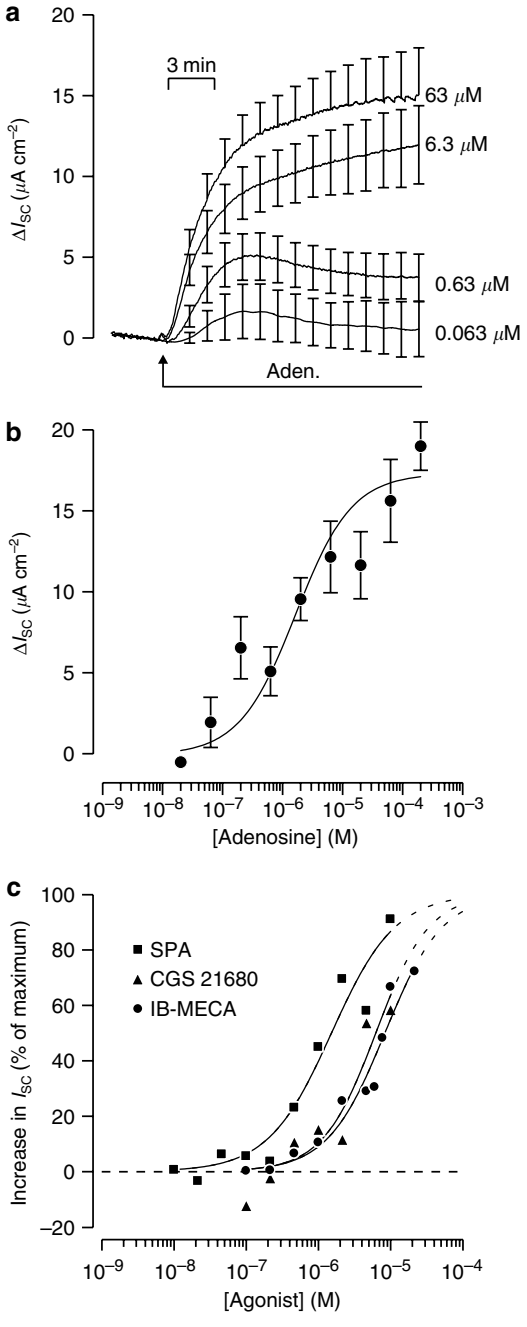

Figure 9 Effects of adenosine and adenosine receptor agonists upon I_{SC}. (**a**) I_{SC} was recorded during stimulation with apical adenosine: 0.063 μM, $n=3$, basal $I_{SC}=27.2\pm3.9\,\mu A\,cm^{-2}$; 0.63 μM, $n=7$, basal $I_{SC}=33.4\pm3.8\,\mu A\,cm^{-2}$; 6.3 μM $n=7$, basal $I_{SC}=25.7\pm5.4\,\mu A\,cm^{-2}$; or 63 μM $n=6$, basal $I_{SC}=27.2\pm3.9\,\mu A\,cm^{-2}$. All data are shown as mean$\pm$s.e.m. and the basal I_{SC} (i.e. that measured before the addition of adenosine) was subtracted from all records in order to illustrate the adenosine-evoked changes in I_{SC}. (**b**) The increases in I_{SC} measured after $\sim$20 min exposure to apical adenosine (0.021–200 μM) were measured and plotted (mean$\pm$s.e.m.) against the concentration of adenosine used. (**c**) Cells were exposed to different concentrations of apical SPA, CGS 21680 or IB-MECA and the resultant increases in I_{SC} measured and normalized to the response to a maximally effective concentration (200 μM) of apical adenosine measured in age-matched cells at identical passage. The results of this analysis are plotted against the concentration of agonist used; all data points are the mean values and error bars have been omitted in the interests of clarity. In all experiments, the cultured epithelial cell layers were each exposed to only a single concentration of agonist, and mean values derived from at least three independent experiments. The solid curves were fitted (least-squares regression) to the experimental data by assuming that the tested drugs bind to a single site as effectively as adenosine. Dashed lines denote extrapolation beyond the range of observed values.

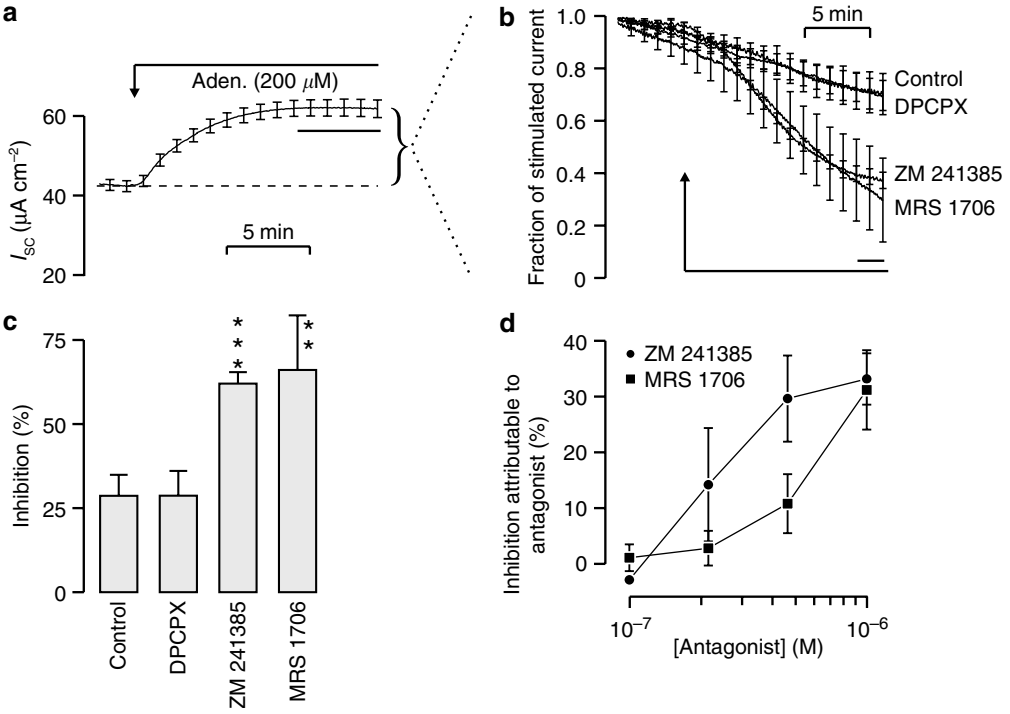

Figure 10 Effects of adenosine receptor antagonists on I_{SC}. (**a**) Time course showing the effects of apical adenosine (200 μM) upon I_{SC} (mean $\pm$ s.e.m., $n=20$). Curved bracket indicates the adenosine-evoked increase in I_{SC}, which was determined for each individual experiment. (**b**)The effects of DMSO solvent vehicle (control, $n=5$), DPCPX (1 μM, $n=5$), ZM 241385 (1 μM, $n=5$) and MRS 1706 (1 μM, $n=5$) on the adenosine-evoked increase in I_{SC}. The bar indicates the period during which data were used to analyse inhibitory effects of the compounds. (**c**) The inhibitory effects of these compounds, presented as mean $\pm$ s.e.m.; asterisks denote values that differ significantly from control (***$P<0.0001$, **$P<0.002$). (**d**) Results of experiments in which this protocol was used to quantify the inhibitory actions of different concentrations (0.1–1 μM) of ZM 1706 and of MRS 241385. The inhibitory effect of the solvent vehicle was monitored in each experiment so that could be corrected for the spontaneous/solvent-evoked fall in I_{SC}.

these data support the notion that the response to purine nucleotides includes a component mediated via adenosine receptors, the unexpected difference between different batches of ATP-γ-S was surprising. Subsequent analysis showed that the second batch used actually contained substantial amounts of ADP, and, although ATP-γ-S is not subject to hydrolysis, the contaminant would be rapidly converted to adenosine (see e.g. Huang *et al*., 2001; Lazarowski *et al*., 2004). Such contamination of commercially available nucleotides has been noted previously and significantly delayed a proper understanding of P2Y receptor pharmacology (see e.g. Nicholas *et al*., 1996; Wilson *et al*., 1998).

Pharmacological basis of the response to adenosine

Apical adenosine caused a slowly developing and sustained increase in I_{SC} similar to that seen during exposure to ATP, and cross-desensitization experiments established that prestimulation with adenosine selectively inhibited the sustained component of the response to ATP. Moreover, prestimulation with ATP reduced, but did not abolish, the response to adenosine, and, taken together, these data make it very likely that the anomalous response to ATP is actually mediated by adenosine. We cannot, however, exclude the possibility that at least part of this response may reflect the presence of adenosine as a contaminant of ATP, but this seems unlikely as basolateral adenosine increased I_{SC} while basolateral ATP did not. This finding implies that ATP hydrolysis occurs only at the apical membrane, a conclusion that accords with previous data (Lazarowski *et al*., 2004).

Subsequent experiments aimed to establish the pharmacological properties of the receptor population underlying this adenosine-evoked increase in I_{SC}. Our initial attempts to construct cumulative concentration–response curves for adenosine receptor agonists and antagonists, which would have allowed the clearest insight into the pharmacology of the receptors, were unsuccessful. We were therefore forced to adopt an alternative strategy in which each cultured epithelial monolayer was exposed to only a single concentration of a particular drug. Although there was variability in the data obtained in this way, these experiments showed clearly that the response to adenosine was concentration dependent and established that SPA, CGS 21680 and IB-MECA could all mimic this response. Analysis of these data indicated that the rank order of potency for the tested compounds was adenosine $\approx$SPA$>$CGS 21680 $\approx$IB-MECA and, as the latter compounds display relatively high affinities for A_1, A_{2A} and A_3 receptors, respectively (Ralevic and Burnstock, 1998; Fredholm *et al*., 2001), the fact that none were more potent than adenosine suggest that these receptors do not make a major contribution to the present response.

By inference, these data thus suggest that the increase in I_{SC} is mediated via A_{2B} receptors, but this was difficult to test directly as there are, at present, no selective agonists for this receptor subtype. We therefore investigated the extent to which adenosine receptor antagonists could reverse the adenosine-evoked increase in I_{SC}. These studies showed that DPCPX, a relatively selective A_1 receptor antagonist, had no effect upon the response to a maximally effective concentration of adenosine, providing evidence against a major role for these receptors. On the other hand, ZM 241385 and MRS 1706, which block A_{2A} and A_{2B} receptors, respectively, both inhibited the functional response to adenosine. Although we explored the kinetics of these inhibitory effects, we could not discern any unambiguous difference between the compounds and so these studies suggest that the response is mediated by an unidentified A_2 receptor subtype and, as mRNA encoding both A_{2A} and A_{2B} receptors was present, it is entirely possible that both may contribute to the response. It is interesting, however, that DPCPX inhibited the sustained component of the response to ATP, but had no effect upon the response to adenosine. The simplest explanation of this is that DPCPX may be unable to antagonize the response to a maximally effective concentration of adenosine, but can block the responses to lower concentrations produced by local hydrolysis of ATP. However, as A_1 receptor mRNA was present, it is also possible that concomitant activation of $P2Y_2$ receptors, which will occur during exposure to ATP, may facilitate signalling via these receptors.

Physiological basis of the response to adenosine

The present responses to adenosine were blocked by amiloride and involved clear increases in the apical Na^+ conductance and these findings indicate that activation of $A_{2A/B}$ receptors leads to a stimulation of Na^+ transport in H441 cells. Although such receptors have previously been described in airway epithelia, previous data suggest that their physiological role is to control the secretion of anions, and hence liquid, into the luminal fluid and this response is thought to reflect activation of cystic fibrosis transmembrane conductance regulator (CFTR) (Lazarowski *et al*., 1992; Clancy *et al*., 1999; Huang *et al*., 2001; Cobb *et al*., 2002, 2003). It is therefore relevant that at least one previous study of H441 cells has described high levels of CFTR expression and shown that cyclic adenosine monophosphate (cAMP)-coupled agonists can increase membrane Cl^- conductance (Kulaksiz *et al*., 2002). However, this finding is at variance with data from several subsequent studies, which have independently shown that H441 cells are absorptive and that activation of cAMP/protein kinase A (PKA) causes a rise in G_{Na} (Sayegh *et al*., 1999; Lazrak and Matalon, 2003; Clunes *et al*., 2004; Ramminger *et al*., 2004; Shlyonsky *et al*., 2005). We can offer no explanation for this discrepancy, although it might be relevant that Kulaksiz *et al*. (2002) did not actually explore the effects of withdrawing external Na^+ and thus failed to quantify the membrane Na^+ current, which would almost certainly have contributed to the current records shown in their paper (see Clunes *et al*., 2004; Shlyonsky *et al*., 2005). Moreover, our own data show that cAMP-elevating compounds selectively increase the amiloride-sensitive component of the membrane conductance, suggesting that any activation CFTR is minimal (Clunes *et al*., 2004).

As far as we are aware, the present data thus provide the first description of adenosine-evoked Na^+ transport in airway epithelial cells, although A_1 receptor antagonists have been reported to cause natriuresis and diuresis *in vivo* (Wilcox *et al*., 1999) and adenosine appears to activate an amiloride-sensitive Na^+ conductance in human intestinal epithelia (Bouritius and Groot, 1997) and stimulate Na^+ transport in a renal cell line (A6). The responses seen in A6 cells appear to reflect an A_1 receptor-mediated rise $[Ca^{2+}]_i$ (Lang *et al*., 1985; Hayslett *et al*., 1995; Macala and Hayslett, 2002), but this is unlikely to explain the response described here as the receptors underlying the present response are, at best, only weakly coupled to $[Ca^{2+}]_i$ and as DPCPX had no effect upon the response to adenosine. Moreover, A_{2B} and A_{2A} receptors characteristically signal via cAMP/PKA (Ralevic and Burnstock, 1998; Fredholm *et al*., 2001) and previous studies of H441 cells have shown that the activation of this signalling pathway can lead to an increase in G_{Na} and a consequent stimulation of Na^+ transport (e.g. Morris and Schafer, 2002; Lazrak and Matalon, 2003; Clunes *et al*., 2004; Ramminger *et al*., 2004). The present response to adenosine was clearly associated with a rise in G_{Na}. It is now clear that cAMP-coupled agonists exert control over G_{Na} by inactivating a mechanism that normally drives the continual removal of Na channel complexes from the apical membrane (Canessa *et al*., 1993, 1994; Debonneville *et al*., 2001; Snyder *et al*., 2001, 2002, 2004; Wang *et al*., 2001; Kamynina and Staub, 2002; McDonald *et al*., 2002; Friedrich *et al*., 2003). However, we have recently shown that adenosine also activates basolateral K^+ channels in H441 cells (Inglis and Wilson, 2006) and, although the molecular basis of this response is unknown, the resultant rise in K^+ conductance may contribute to adenosine-evoked Na^+ transport by hyperpolarizing the membrane potential and increasing the driving force for Na^+ entry (see Gordon and MacKnight, 1991). The adenosine-evoked stimulation of Na^+ transport described here is thus a complex process and this complexity may, at least in part, explain why it was so difficult to discern the pharmacological properties of the present responses as it makes a simple relationship between fractional receptor occupancy and the rate of Na^+ transport highly unlikely.

H441 epithelial cells are derived from the human distal airways and have been reported to display morphological and functional features of Clara cells, secretory cells found in the distal airways (O'Reilly *et al*., 1989; Kulaksiz *et al*., 2002). Electrophysiological studies, on the other hand, have shown that these cells express an absorptive phenotype and they have thus become a widely accepted model of the Na^+ absorbing surface epithelium of the human distal airway (Sayegh *et al*., 1999; Lazrak and Matalon, 2003; Clunes *et al*., 2004; Ramminger *et al*., 2004; Shlyonsky *et al*., 2005; Woollhead *et al*., 2005). The present data, in common with previous (see e.g. Inglis *et al*., 1999, 2000; Ramminger *et al*., 1999; Cuffe *et al*., 2000; Kunzelmann and Mall, 2003; Kunzelmann *et al*., 2005), show nucleotides can cause sustained inhibition of this ion transport process by activating $P2Y_2$ receptors. However, ATP is subject to rapid

extracellular hydrolysis (Lazarowski *et al*., 2004) to adenosine and it is now clear that this metabolite is capable of inducing a sustained stimulation of Na^+ absorption that could lead to dehydration of the airway surface liquid (see, e.g. Boucher, 2004). The present data thus draws attention to the possibility that adenosine may play a previously unrecognized role in airway fluid homeostasis and makes exploring the effects of adenosine receptor agonists/antagonists upon fluid homeostasis in freshly isolated human distal airway epithelial cells important.

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Conflict of interest

The authors state no conflict of interest.

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