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$P2Y_2$ receptor-mediated inhibition of ion transport in distal lung epithelial cells

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1 Rat foetal distal lung epithelial cells were plated onto permeable supports where they became integrated into epithelial sheets that spontaneously generated short circuit current (I_{SC}).

2 Apical ATP (100 μM) evoked a transient fall in I_{SC} that was followed by a rise to a clear peak which, in turn, was succeeded by a slowly developing decline to a value below control. Apical UTP evoked an essentially identical response.

3 UDP and ADP were ineffective whilst ATP had no effect when added to the basolateral solution. These effects thus appear to be mediated by apical $P2Y_2$ receptors.

4 The rising phase of the responses to ATP/UTP was selectively inhibited by anion transport inhibitors but persisted in the presence of amiloride, which abolished the inhibitory effects of both nucleotides. Thus, apical nucleotides appear to evoke a transient stimulation of anion secretion and sustained inhibition of Na^+ absorption.

5 Basolateral isoprenaline (10 μM) elicited a rise in I_{SC} but subsequent addition of apical ATP reversed this effect. Conversely, isoprenaline restored I_{SC} to its basal level following stimulation with ATP. Apical $P2Y_2$ receptors and basolateral β-adrenoceptors thus allow their respective agonists to exert mutually opposing effects on I_{SC}.

Keywords: $P2Y_2$ receptors; FDLE cells; pulmonary ion transport; Ussing chambers; Na^+ transport; epithelial Na^+ channel

Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; DMEM, Dulbeco's modified Eagle's medium; DPC, diphenylamine-2-carboxylic acid; FDLE cells; foetal distal lung epithelial cells; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulphononic acid; I_{SC}, short circuit current; *p.d.*, transepithelial potential difference; R_t, transepithelial resistance

Introduction

Throughout foetal life, the distal lung epithelia secrete fluid into the developing airspaces and establish a distending pressure crucial to proper lung morphogenesis. However, this liquid must be removed from the lungs if the new-born infant is to breathe air at birth and so, during the late stages of pregnancy, the alveolar epithelia undertake the net absorption of fluid and remove this liquid. It is now clear that the secretion seen in the foetal lung depends upon the transport of anions into the lung lumen (Olver & Strang, 1974) whilst the subsequent removal of this fluid is driven by the active transport of Na^+ from the lung liquid. During labour, a rise in foetal adrenaline appears to stimulate this process by activating alveolar β_2-receptors (Olver & Strang, 1974; Walters & Olver, 1978; Brown *et al*., 1983; Olver *et al*., 1986; O'Brodovich, 1991; Tohda *et al*., 1994; Matthay *et al*., 1996; Barker, 1997; Ito *et al*., 1997). However, in addition to their β_2-receptors, the distal rat lung epithelia express $P2Y_2$ receptors (Rice *et al*., 1995; Clunes *et al*., 1998). These are activated, with equal efficacy and potency, by ATP and uridine 5′ triphosphate (UTP) but are insensitive to nucleotide disphosphates (Nicholas *et al*., 1996). Although the physiological role of these receptors is unknown, $P2Y_2$ receptors are also found in the epithelia of the proximal airways where they allow nucleotides to control epithelial ion transport processes (see e.g. Mason *et al*., 1991; Knowles *et al*., 1996). In the present study, we therefore explore the possibility that the $P2Y_2$ receptors expressed by rat foetal distal lung epithelial (FDLE) cells may also allow control over epithelial ion transport.

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Methods

Solutions and chemicals

Bicarbonate-buffered physiological saline (mM): NaCl, 117; $NaHCO_3$, 25, KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5 and D-glucose, 11; pH 7.3–7.4 when bubbled with 5% CO_2/95% O_2. 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES)-buffered saline (mM): NaCl, 120; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1; D-Glucose, 10; HEPES, 20; pH adjusted to 7.4 with NaOH. UTP and ATP were obtained from Pharmacia (St. Albans, Herts, U.K.) as ultrapure solutions (100 mM) and stored at −20°C; independent analysis has shown that these nucleotides, in contrast to those from many other sources (see e.g. Nicholas *et al*., 1996), are essentially pure (R.C. Boucher, personal communication). UDP and ADP (Sigma, Poole, Dorset, U.K.) were prepared from 10 mM stock solutions (stored at −20°C) that had been preincubated (1 h, 37°C) in HEPES-buffered physiological saline containing hexokinase (10 i.u. ml^{-1}, Boehringer) and 22 mM D-glucose in order to convert contaminating nucleotide triphosphates to the respective diphosphates (Nicholas *et al*., 1996). Amiloride and isoprenaline were freshly prepared (10 mM in distilled water) on each experimental day whilst stock solutions of dipheny-

lamine-2-carboxylic acid (DPC, Aldrich, Gillingham, Dorset, U.K., 10 mM), the acetoxymethylester (AM) form of Fura-2 (Molecular Probes, Eugene, OR, U.S.A., 2 mM) and bumetanide (Sigma, 10 mM) were prepared in dimethyl sulphoxide, aliquoted and stored at −20°C. Appropriate experiments showed that the solvent vehicle did not influence I_{SC}. Cell culture reagents were from Paisley Life Technologies and general laboratory reagents were from Sigma.

Isolation and culture of FDLE cells

Foetuses from anaesthetized (3% Halothane) 20 day pregnant (term = 22 days) rats were decapitated and their lung tissue collected into ice-cold, Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution, chopped into pieces (<0.5 mm) and digested with 0.2% trypsin/0.012% DNAse (2 × 20 min, 37°C) followed by 0.1% collagenase/0.012% DNAse (15 min, 37°C). Both enzyme solutions were prepared in Dulbecco's modified Eagle's medium (DMEM). The resultant digest was washed by centrifugation (400 × *g*, 5 min)/resuspension in fresh medium, and the cells then incubated in a culture flask for 1 h. The supernatant was then gently decanted in order to separate non-adherent epithelial cells from fibroblasts and smooth muscle cells which characteristically attach rapidly to plastic. After a second such fractionation, the non-adherent cells were washed by repeated (four times) centrifugation (130 × *g*, 3 min)/resuspension in fresh medium, and finally resuspended in DMEM containing 10% foetal bovine serum. Previous work showed that essentially all cells isolated in this way are of epithelial lineage and that fibroblast contamination is negligible (Clunes *et al*., 1998). For experiments, cells were plated (10^6 cells cm^{-2}) onto Transwell Col membranes (Costar, High Wycomb, Bucks, U.K.) and incubated for 24 h. Non-viable cells were then removed by gently washing each culture and the remaining cells incubated in serum-free medium PC-1 for 24 h. After this time they had consistently become integrated into coherent epithelial sheets. All incubations were carried out at 37°C in an atmosphere of water-saturated room air containing 5% CO_2. The ambient O_2 tension (PO_2) was thus ~152 mmHg.

Quantification of transepithelial ion transport

Transwell Col membranes bearing FDLE cells were mounted in Ussing chambers and bathed with bicarbonate-buffered saline. Each preparation was first maintained under open circuit conditions whilst the transepithelial potential difference (*p.d.*) was monitored. Once this settled to a stable value (30–40 min), the *p.d.* was clamped to 0 mV and the current required to hold this potential (short circuit current, I_{SC}) was digitized (4 Hz) and recorded directly to computer disk using a PowerLab computer interface and associated software (ADI Instruments, Hastings, East Sussex, U.K.). Transepithelial resistance (R_t) was monitored at the beginning and end of each experiment by measuring the currents flowing in response to brief excursions (1 mV) from this holding potential.

Quantification of changes in $[Ca^{2+}]_i$

FDLE cells on Transwell Col membranes were loaded with the Ca^{2+}-sensitive fluorescent dye Fura-2 by incubation (20–40 min) in medium containing the dye's membrane-permeant AM form (4 μM). A rise in $[Ca^{2+}]_i$ causes a corresponding rise in the Fura-2 fluorescence ratio recorded from cells loaded with this dye, which allows changes in $[Ca^{2+}]_i$ to be recorded (Grynkiewicz *et al*., 1985). Membranes bearing Fura-2 loaded epithelia were therefore mounted in a home-made holder attached to the stage of a Nikon inverted microscope where the apical (i.e. upward facing) surface of the cell layer was superfused with HEPES-buffered saline. Fura-2 fluorescence ratios (excitation wavelengths 340 nm and 380 mm) were recorded at 1 Hz from an optical field containing 30–50 cells, digitized (1 Hz) and recorded to computer disk.

Experimental design and data analysis

Positive currents are defined as those carried by anions moving from the basolateral to the apical bath and are displayed as upward deflections of the traces. Control I_{SC} was defined as the current recorded immediately after the epithelia were voltage clamped. In order to facilitate analysis, all data were normalized to the magnitude of this initial current. The times at which the epithelia were exposed to various drugs were carefully logged so that the data points defining the current records could be accurately aligned. This permitted calculation of a series of mean values that are displayed ± s.e.mean. A similar approach was used to display Fura-2 fluorescence ratios. These manipulations were undertaken using the standard features of a commercially available spreadsheet package (Microsoft Excel 97). Experimentally-induced changes in the I_{SC} were quantified by measuring the current flowing at the peak of a response and subtracting the current flowing measured prior to an experimental manipulation. Such data are presented as mean ± s.e.mean and, unless otherwise stated, the statistical significance of any differences between these mean values was evaluated using Student's paired *t*-test.

Results

When cultured on Transwell Col membranes, FDLE cells consistently became integrated into epithelial monolayers (R_t > 200 Ωcm^2) that spontaneously generated I_{SC}. To explore the extent to which this basal current was maintained, I_{SC} was recorded whilst epithelia (n = 4) were bathed with control saline. At the onset of these experiments, R_t and I_{SC} were 415 ± 18 Ωcm^2 and 8.8 ± 3.7 μA cm^{-2} respectively, and these parameters both remained within ±5% of their respective initial values throughout a 90 min incubation (final values: R_t, 418 ± 16 Ωcm^2, I_{SC}, 8.9 ± 3.6 μA^{-2}). We therefore conclude that spontaneous changes in I_{SC} do not occur over the time scale of the present experiments.

Effects of nucleotides upon I_{SC}

Apical ATP (100 μM) evoked a complex series of changes in I_{SC} but, in each preparation, the response could be resolved into three components. Initially there was a sharp fall in I_{SC} that occurred with no discernible latency but this was superseded by a slower rise which persisted for 2–3 min, by which time I_{SC} had reached a clearly defined peak elevated above the basal value (Figure 1a). Thereafter, there was a slow but persistent fall until, after ~40 min exposure to ATP, I_{SC} had declined to ~50% of its initial value. Apical UTP elicited essentially identical changes in I_{SC} (Figure 1a, Table 1). I_{SC} did not change during exposure (30 min) to apical ADP (100 μM, n = 6, control I_{SC} = 22.1 ± 6.3 μA cm^{-2}, initial R_t = 281 ± 39 Ωcm^2, final R_t = 285 ± 38 Ωcm^2) or UDP (100 μM, n = 4, control I_{SC} = 20.1 ± 6.6 μA cm^{-2}, initial R_t = 255 ± 35 Ωcm^2, final R_t = 250 ± 41 Ωcm^2). Moreover, 100 μM ATP failed to

evoke any response when added to the basolateral solution ($n=3$, R_t not recorded).

Effects of ion transport inhibitors upon the responses to ATP and UTP

Figure 1b shows the effects of apical ATP and UTP (both 100 μM) upon epithelia that had been pre-treated with apical DPC (1 mM) and basolateral bumetanide (10 μM), an experimental situation in which transepithelial anion secretion would be prohibited. Basal I_{SC} was $\sim$80% of its control value but epithelia that had been treated in this way continued to respond to the nucleotides. During stimulation with ATP, the initial fall in I_{SC} could not be distinguished from control but the subsequent rise was attenuated so that the current now failed to rise above the level recorded prior to adding ATP. However, the slowly developing fall in I_{SC} persisted and could not be distinguished from control (Figure 1b, Table 1). The cultured epithelia also responded to apical UTP under these conditions (Figure 1b) and analysis of the data failed to reveal any statistically significant differences between the responses seen in ATP- and UTP-stimulated cells (Table 1). Pre-treating the epithelia with apical amiloride (10 μM), a substance that blocks epithelia sodium channels, caused basal I_{SC} to fall to $\sim$20% of its control value (Figure 1c). Whilst amiloride-treated cells responded to apical ATP or UTP (Figure 1c) the responses now consisted of increases to a peak value. Thereafter, the current then fell slowly back towards its basal value. There was no evidence of the fall below the basal level that was seen in control epithelia (Table 1).

ATP-evoked changes in $[Ca^{2+}]_i$

The data presented in Figure 2a show that apical ATP increased $[Ca^{2+}]_i$ in the cultured epithelia. This response consisted of a rapid rise to a peak value that was followed by a rapid decline that occurred despite the continued presence

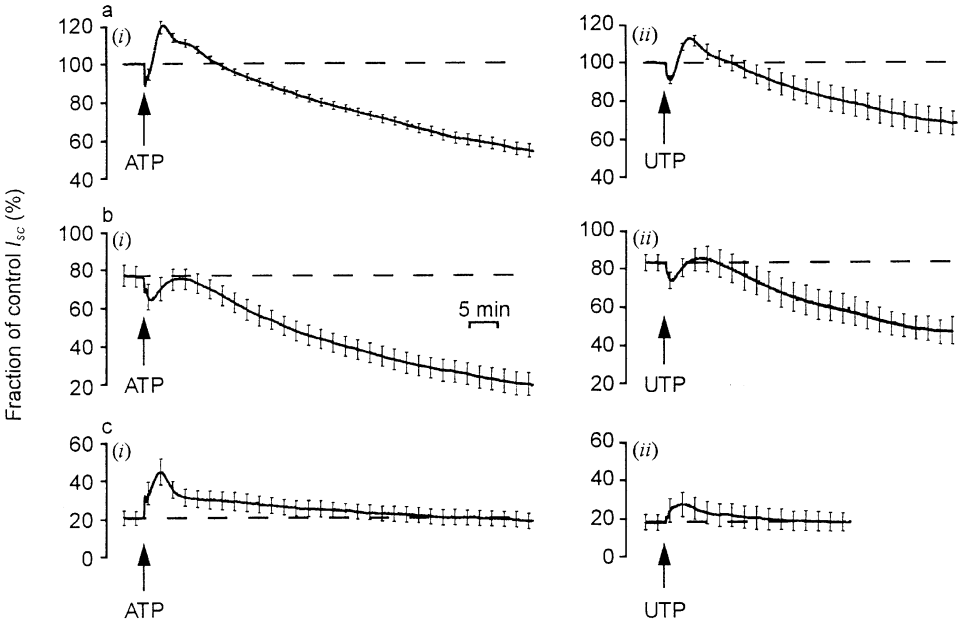

Figure 1 Effects of nucleotides upon I_{sc}. Each panel shows the changes in I_{sc} (mean$\pm$s.e.mean) evoked by adding 100 μM ATP (*i*) or 100 μM UTP (*ii*) to the apical solution. Experiments were undertaken using epithelia that had either been maintained under control conditions (a: ATP, $n=8$, control $I_{sc}=11.0\pm2$ μA cm^{-2}, initial $R_t=373\pm41$ Ωcm^2, final $R_t=375\pm39$ Ωcm^2; UTP, $n=5$, control $I_{sc}=9.7\pm0.8$ μA cm^{-2}, initial $R_t=522\pm74$ Ωcm^2, final $R_t=510\pm78$ Ωcm^2); pre-treated with 1 mM apical DPC and 10 μM basolateral bumetanide: (b: ATP, $n=5$, control $I_{sc}=13.3\pm3.7$ μA cm^{-2}, initial $R_t=312\pm49$ Ωcm^2, final $R_t=240\pm23$ Ωcm^2; UTP, $n=4$, control $I_{sc}=9.8\pm1.7$ μA cm^{-2}, initial $R_t=308\pm54$ Ωcm^2, final $R_t=310\pm53$ Ωcm^2), pre-treated with 10 μM apical amiloride (c: ATP, $n=7$, control $I_{sc}=9.8\pm1.2$ μA cm^{-2}, initial $R_t=366\pm28$ Ωcm^2, final $R_t=372\pm26$ Ωcm^2; UTP, $n=5$, control $I_{sc}=9.5\pm1.3$ μA cm^{-2}, initial $R_t=462\pm59$ Ωcm^2, final $R_t=450\pm57$ Ωcm^2).

Table 1 Effects of ion inhibitors upon the responses to ATP and UTP

	Control cells			*DPC/Bumetanide-treated cells*			*Amiloride-treated cells*		
	Transient fall ($\Delta\mu$A cm^{-2})	*Transient rise* ($\Delta\mu$A cm^{-2})	*Sustained fall* ($\Delta\mu$A cm^{-2})	*Transient fall* ($\Delta\mu$A cm^{-2})	*Transient rise* ($\Delta\mu$A cm^{-2})	*Sustained fall* ($\Delta\mu$A cm^{-2})	*Transient fall* ($\Delta\mu$A cm^{-2})	*Transient rise* ($\Delta\mu$A cm^{-2})	*Sustained fall* ($\Delta\mu$A cm^{-2})
ATP	-0.9 ± 0.3*	1.5 ± 0.2***	-4.6 ± 0.8***	-1.5 ± 0.3**	-0.4 ± 0.6	-7.3 ± 1.8****	–	2.3 ± 0.3***	-0.1 ± 0.2
UTP	-0.9 ± 0.2*	1.4 ± 0.3***	-3.3 ± 0.6***	-1.0 ± 0.3*	0.6 ± 0.3	-3.7 ± 0.8*	–	1.1 ± 0.3*	0.1 ± 0.1

Epithelia were stimulated with 100 μM apicial ATP or UTP and the values of I_{SC} seen during each of the three phases were recorded. The current measured immediately before the cells were exposed to nucleotides also measured and subtracted from each of these values in order to quantify all three phases of the response. This analysis was undertaken for control epithelia; epithelia that had been pre-treated with 1 mM apical DPC and 10 μM basolateral bumetanide, or with 10 μM apical amiloride. The resultant data are tabulated as mean$\pm$s.e.mean. The statistical significance of the changes in I_{SC} was assessed by using Student's paired *t*-test to compare the currents measured before and after stimulation. The results of this analysis are indicated by asterisks (*$P<0.05$, **$P<0.02$, ***$P<0.01$, ****$P<0.005$).

of agonist. $[Ca^{2+}]_i$ had invariably returned to its basal value after 2 min exposure to ATP. The data in Figure 2b show the ATP-evoked changes in I_{SC}, and have been re-plotted from Figure 1a so that they are now shown on the same time scale used to display the $[Ca^{2+}]_i$ signals. It is clear that the ATP-evoked changes in I_{SC} take place over a much longer time scale than the changes in $[Ca^{2+}]_i$. Indeed, only the initial, rapid fall in I_{SC} is coincident with the rise in $[Ca^{2+}]_i$.

Effects of thapsigargin upon the response to nucleotides

To explore the effects of thapsigargin upon the ability of nucleotides to evoke changes in I_{SC}, this drug (0.3 μM) was first added to the solution bathing both sides of the cultured epithelia. This caused I_{SC} to fall from 19.8 ± 2.0 μA cm^{-2} to 18.6 ± 1.8 μA cm^{-2} over a 12.5 min period (5.4 ± 1.6% inhibition, $n=16$, $P<0.005$) although, in some instances, this was preceded by a barely discernible, transient rise. Subsequently, these thapsigargin-treated cells were either maintained under standard conditions ($n=5$), or also exposed to 100 μM apical ATP ($n=6$) or UTP ($n=5$). In the absence of nucleotides, the thapsigargin-evoked fall in I_{SC} continued (Figure 3a) until, after 1 h, the current had decayed by 30.6 ± 6.7% ($P<0.001$). It was clear, however, that the thapsigargin-treated cells responded to ATP (Figure 3b) and UTP (Figure 3c) with transient falls in I_{SC} (ATP: -1.5 ± 0.2 μA cm^{-2}, $P<0.001$; UTP: -1.6 ± 0.3 μA cm^{-2}, $P<0.05$) that did not differ significantly from those seen in control cells (Table 1). These initial responses were succeeded by slowly developing increases to peak values that were elevated above the level recorded immediately before the cells were exposed to nucleotides (ATP: 0.4 ± 0.1 μA cm^{-2}, $P<0.05$; UTP: 0.5 ± 0.2 μA cm^{-2}, $P<0.05$). However, these increases were smaller (ATP: $P<0.001$, UTP: $P<0.05$) than the equivalent control responses (Table 1). Once these initial changes in I_{SC} had subsided, the current continued to fall in the nucleotide-stimulated cells and analysis showed that the total decline seen over a 1 h period in ATP- (38.5 ± 9.9%) and UTP-stimulated cells (53.7 ± 13.2%) did not differ significantly from that seen in cells that had been simply exposed to thapsigargin. Separate studies in Fura-2 loaded epithelia showed that thapsigargin (0.3 μM) elicited a clear but transient rise in $[Ca^{2+}]_i$ in only three out of 17 preparations. However, subsequent application of apical ATP consistently failed to elicit a discernible rise in $[Ca^{2+}]_i$ in thapsigargin-treated epithelia ($n=7$).

Effects of isoprenaline

Basolateral isoprenaline (10 μM) elicited an initial, complex series of changes in I_{SC} that gave way to a slow but steady rise until, after $\sim$30 min, the current had risen to a value that was 93.4 ± 1.7% above ($P<0.005$, paired t-test) control (Figure 4a). Separate experiments ($n=3$, control $I_{SC}=19.6\pm2.7$ μA cm^{-2}, initial $R_t=445\pm14$ Ωcm^2, final $R_t=455\pm17$ Ωcm^2), showed that phenylephrine had no discernible effect whilst pre-treatment with amiloride (10 μM) essentially abolished the

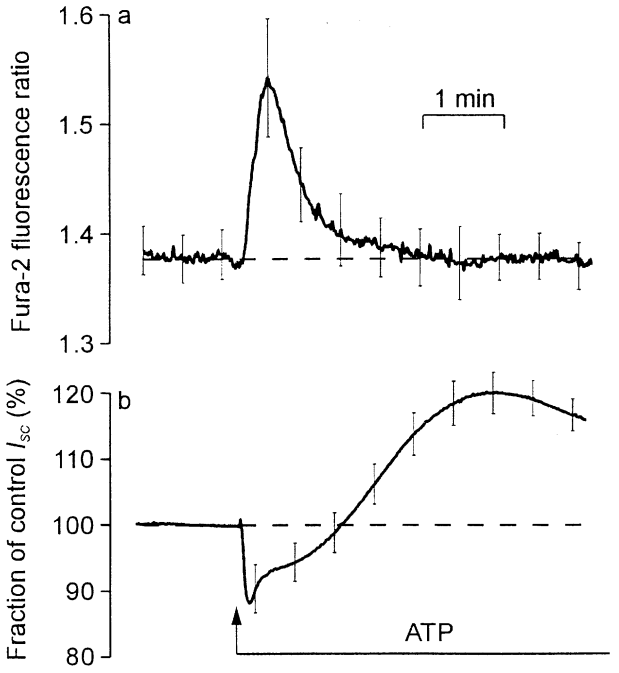

Figure 2 ATP-evoked $[Ca^{2+}]_i$-signals. (a) Fura-2-fluorescence ratios were recorded from groups of 30–50 cells that formed part of a coherent layer of FDLE cells cultured on Transwell Col membranes. The epithelia were exposed to 100 μM apical ATP as indicated by the arrows. Data ($n=5$) were pooled to obtain an average trace. Vertical bars show $\pm$s.e.mean. (b) The ATP evoked changes in I_{sc} that are shown in Figure 1a are replotted (mean$\pm$s.e.mean) using an expanded timescale to allow direct comparison with the ATP-evoked changes in $[Ca^{2+}]_i$.

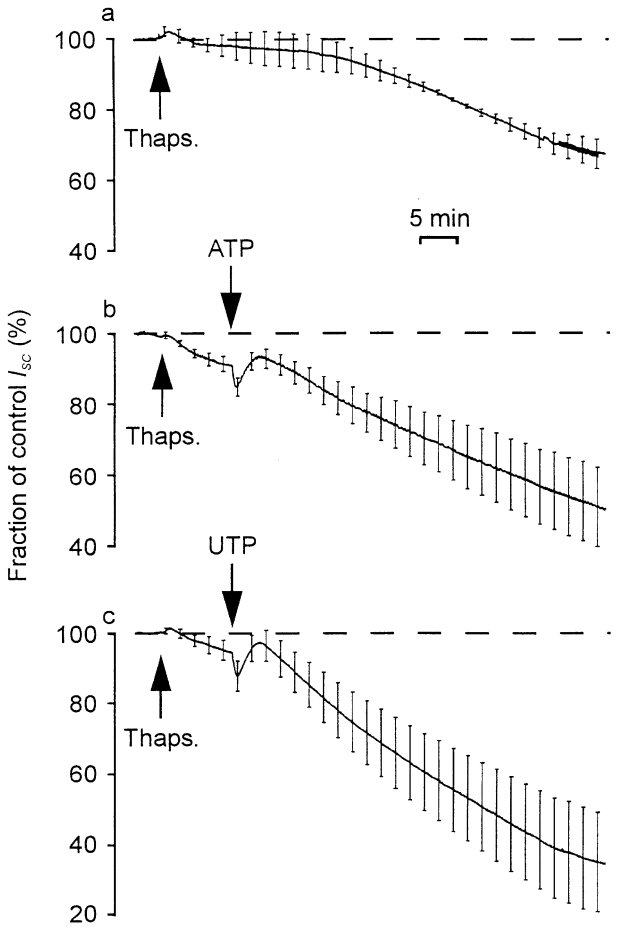

Figure 3 Effects of thapsigargin upon the nucleotide-evoked changes in I_{sc}. (a) The effects of 0.3 μM thapsigargin ($n=5$, control $I_{sc}=13.9\pm4.7$ μA cm^{-2}, initial $R_t=430\pm71$ Ωcm^2, final $R_t=395\pm67$ Ωcm^2). (b) The effects of exposing the epithelia to 0.3 μM thapsigargin followed by 100 μM apical ATP ($n=6$, control $I_{sc}=23.0\pm2.2$ μA cm^{-2}, initial $R_t=430\pm86$ Ωcm^2, final $R_t=465\pm93$ Ωcm^2). (c) The effects of exposing the epithelia to 0.3 μM thapsigargin followed by 100 μM apical UTP ($n=5$, control $I_{sc}=22.0\pm2.3$ μA cm^{-2}. Initial $R_t=345\pm30$ Ωcm^2, final $R_t=345\pm38$ Ωcm^2).

response to the subsequent application of 10 μM isoprenaline (Figure 4b, 93.4$\pm$2.7% inhibition, $n=8$, $P<0.02$, Student's unpaired t-test).

Interactions between ATP and isoprenaline

To explore the possibility that apical nucleotides might modify the response to basolateral isoprenaline, experiments were undertaken in which ATP (100 μM) was added to the apical solution once the response to isoprenaline was fully established. As anticipated, isoprenaline elicited an initial series of transient changes in I_{SC} that was followed by a slowly-developing rise until, after 20–25 min, I_{SC} had risen to a value that was ~70% greater than control (Figure 5a). Subsequent addition of ATP elicited an initial transient that was followed by a slowly-developing decline until, after 20–25 min, I_{SC} had fallen to a value that did not differ significantly from control. Whilst these data suggest that apical ATP opposes the stimulatory action of basolateral isoprenaline, they do not unequivocally establish this point as the experimental design does not exclude the possibility that the response to isoprenaline may not be sustained during prolonged stimulation. We therefore carried out further experiments in which the response to isoprenaline was monitored over a 90 min period. In these experiments ($n=5$, initial $R_t=432\pm43$ Ωcm^2, final $R_t=434\pm40$ Ωcm^2) basal I_{SC} was 9.1 ± 1.4 μA cm^{-2} but rose to 20.1 ± 1.3 μA cm^{-2} after 25 min exposure to 10 μM isoprenaline. Moreover, the current was maintained at this elevated level throughout the entire incubation period (I_{SC} after 90 min: 20.0 ± 1.24 μA cm^{-2}). We therefore conclude that the fall in I_{SC} seen in Figure 5a is due to the presence of ATP in the apical solution. The data presented in Figure 5b show the effects of basolateral isoprenaline (10 μM) upon epithelia that had first been stimulated with apical ATP (100 μM). As anticipated, I_{SC} fell by ~45% ($P<0.0001$, paired t-test) after 20–25 min exposure to ATP confirming the inhibitory action of this nucleotide. However, the ATP-stimulated cells subsequently responded to basolateral isoprenaline so that, after a further 25–30 min, I_{SC} had returned to a value that did not differ significantly from control (Figure 5b).

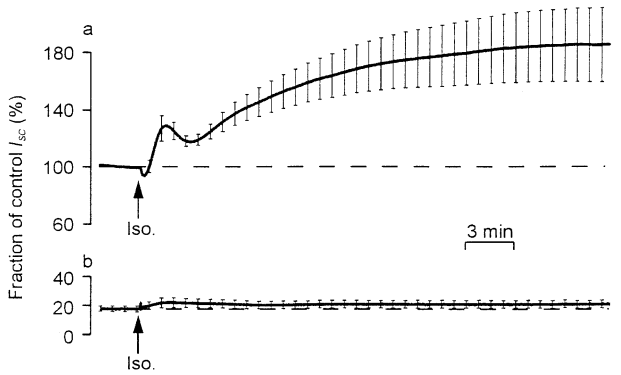

Figure 4 Effects of isoprenaline. Cultured epithelia were stimulated with 10 μM basolateral isoprenaline either (a) under control conditions $n=9$, control $I_{sc}=12.2\pm2.2$ μA cm^{-2}, initial R_t 427 ± 101 Ωcm^2, final $R_t=389\pm82$ Ωcm^2 or (b) after pre-treatment with 10 μM apical amiloride, $n=8$, control $I_{sc}=18.8\pm2.5$ μA cm^{-2}, initial $R_t=320\pm23$ Ωcm^2, final $R_t=331\pm24$ Ωcm^2.

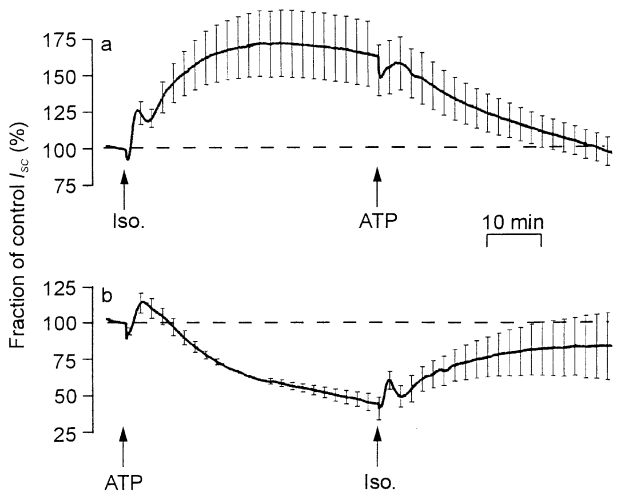

Figure 5 Functional interactions between isoprenaline and ATP. (a) Cultured epithelia ($n=7$, control $I_{sc}=15.3\pm2.9$ μA cm^{-2}, initial $R_t=335\pm42$ Ωcm^2, final $R_t=340\pm44$ Ωcm^2) were stimulated with 10 μM basolateral isoprenaline (Iso.) and 100 μM ATP as indicated. (b) Data from experiments in which the epithelia ($n=4$), control $I_{sc}=7.3\pm1.5$ μA cm^{-2}, initial $R_t=375\pm60$ Ωcm^2, final $R_t=339\pm45$ Ωcm^2) were exposed to 100 μM apical ATP followed by 10 μM basolateral isoprenaline. Data are presented as mean$\pm$s.e.mean.

Discussion

When cultured on permeable supports, FDLE cells consistently become integrated into coherent epithelial layers that spontaneously generate I_{SC}. Our values of R_t, $p.d.$ and I_{SC} generally agree well with those presented in previous studies of these cells (see reviews by O'Brodovich, 1991; Matthay *et al.*, 1996) and experiments using pharmacological inhibitors of ion transport suggest that ~80% of the spontaneous I_{SC} was due to the absorption of Na^+ from the apical solution whilst ~20% was due to the secretion of anions into this liquid. Whilst this is in broad agreement with earlier data, most previous studies suggest that a smaller fraction (15–60%) of the basal I_{SC} is due to Na^+ absorption (see reviews by O'Brodovich, 1991; Matthay *et al.*, 1996). Whilst we can offer no firm explanation for this discrepancy, the gestational age of the donor foetus and the conditions under which cells are cultured are important sources of variability between such studies. Cells isolated early in development display a predominantly secretory phenotype whereas cells from adult animals form cultures that absorb Na^+ in the absence of detectable anion secretion (Rao & Cott, 1991; Barker *et al.*, 1995; Borok *et al.*, 1998a, b; Danto *et al.*, 1998). The cells used in the present study were isolated late in gestation and maintained at a PO_2 of ~152 mmHg. This is higher than that experienced *in utero* and it is has recently become clear that elevated PO_2 stimulates Na^+ absorption in alveolar epithelia. Indeed, it has been proposed that the rise in alveolar PO_2 that occurs at birth is an important stimulus for alveolar maturation, and the mechanism of this effect is currently under investigation (Pitkänen *et al.*, 1996; Haddad & Land, 1999; Ramminger *et al.*, 1999). Although the present experiments were undertaken using cells from late gestation foetuses, the cultured epithelia almost certainly displayed a neonatal phenotype when used in experiments.

Early studies showed that nucleotides evoke surfactant secretion from distal lung epithelial cells (Rice & Singleton, 1986) but the present data, in common with those presented by Barker and colleagues (Barker *et al.*, 1995; Barker & Gatzy, 1998), show that these substances also exert control over ion transport processes in these cells. This control appears to be mediated *via* the P2Y$_2$ receptors that have been identified in these cells (Rice *et al.*, 1995; Clunes *et al.*, 1998) and which now

appear to be located in the apical plasma membrane. Although their physiological role is enigmatic, apical P2Y receptors are found in essentially all polarized epithelia (see e.g. Wong, 1988; Mason *et al*., 1991; Schwiebert *et al*., 1995; Inoue *et al*., 1997; Kerstan *et al*., 1998; Wilson *et al*., 1998; Ko *et al*., 1999). Moreover, even unstimulated epithelial cells appear to release ATP, and possibly other nucleotides, across their apical membranes and a growing body of evidence suggests that the nucleotides released in this way may activate apical P2Y receptors and so exert autocrine control over epithelial function (see e.g. Schwiebert *et al*., 1995; Taylor *et al*., 1998; Schwiebert, 1999). Interestingly, ATP has been detected in rat bronchiolar/alveolar lavage fluid and the inferred concentration in the distal airway surface liquid appears high enough to activate the receptors described in the present study (Rice *et al*., 1989). These nucleotides may therefore exert tonic control over both surfactant secretion and ion transport.

Although ATP and UTP evoked complex responses, the changes in I_{SC} could be resolved into three components: a transient fall, a clearly defined peak and a slowly-developing but persistent fall. Experiments using pharmacological inhibitors of ion transport suggested that the rising phase of the response was due to transient stimulation of anion secretion, which accords with the data from earlier studies (Barker *et al*., 1995; Barker & Gatzy, 1998), but that the slowly-developing fall was due to reduced Na^+ absorption. Although such dual control over anion secretion and Na^+ absorption was noted in an early study of proximal airway epithelia (Mason *et al*., 1991), almost all subsequent studies that explored the effects of nucleotides upon lung/airway epithelia were undertaken under conditions designed to prohibit Na^+ absorption and allow effects upon anion secretion to be studied in isolation (see e.g. Barker *et al*., 1995; Barker & Gatzy, 1998). P2Y receptor-mediated anion secretion attracted this interest as it was thought that it may offer a means to pharmacologically correct the reduced anion secretion seen in the lungs of patients with cystic fibrosis (CF), a potentially lethal genetic disease (see review by Knowles *et al*., 1996). However, a study of human nasal epithelial cells suggested that, at least over a 2 h period, ATP increases the volume of airway surface liquid by reducing the rate of Na^+ absorption rather than by evoking increased anion secretion (Benali *et al*., 1994). Since then, nucleotide-evoked inhibition of Na^+ absorption has been described in the rabbit cortical collecting tubule (Koster *et al*., 1996) and proximal airway (Iwase *et al*., 1997), and in cultured human bronchial epithelia (Devor & Pilewski, 1999; McAulay *et al*, 1999). $P2Y_2$ receptor-mediated inhibition of Na^+ transport may therefore be a widespread, albeit poorly documented, phenomenon. This could have important implications for the clinical usefulness of P2Y receptor agonists, as hyperabsorption of salt, rather than impaired secretion of chloride, now appears to be a major cause of lung pathology in CF (Stutts *et al*., 1995; Knowles *et al*., 1996; Matsui *et al*., 1999).

$P2Y_2$ receptors, in common with all known P2Y receptor subtypes, allow nucleotides to increase $[Ca^{2+}]_i$ by activating phospholipase C (Berridge, 1993; Dubyak & El-Moatassim, 1993; Nicholas *et al*., 1996). As the ion channel species thought to underlie alveolar Na^+ absorption appears to be inhibited by increased $[Ca^{2+}]_i$ (Hummler *et al*., 1996; Ishikawa., 1998) we were tempted to attribute the inhibition of Na^+ transport to increased $[Ca^{2+}]_i$. Support for this came from the observation that thapsigargin, a substance that increases $[Ca^{2+}]_i$ in many different cell types (Takemura *et al*., 1989; Thastrup *et al*., 1990), also caused a slowly developing fall in I_{SC}. However, not all of our data were consistent with this hypothesis. Experiments in which $[Ca^{2+}]_i$ was monitored in Fura-2 loaded epithelia thus showed that the nucleotide-evoked $[Ca^{2+}]_i$ signals occurred over a much shorter time scale than did the rise in I_{SC}. Moreover, these experiments also showed that thapsigargin elicited little change in $[Ca^{2+}]_i$, although it did attenuate the $[Ca^{2+}]_i$ signals evoked by subsequent application of ATP, presumably by preventing the sequestration of Ca^{2+} within agonist-sensitive stores (Takemura *et al*., 1989; Thastrup *et al*., 1990). It thus appears to be the loss of Ca^{2+} from an agonist sensitive store, rather than a rise in $[Ca^{2+}]_i$ *per se*, that underlies the inhibition of Na^+ transport. It is possible that this may affect the buffering of $[Ca^{2+}]_i$ in the small volume of cytoplasm immediately adjacent to the apical membrane. However, experiments in which thapsigargin-treated cells were subsequently exposed to ATP or UTP showed that these substances could still evoke transient changes in I_{SC} even though $P2Y_2$ receptor-mediated $[Ca^{2+}]_i$-signalling was prevented. It thus appears that a $[Ca^{2+}]_i$-independent pathway allows the apical $P2Y_2$ receptors to exert control over ion transport. An essentially identical conclusion was drawn from studies of rabbit cortical tubule cells and proximal airway epithelia. In the cortical cells, the effect appears to be mediated *via* protein kinase C (Koster *et al*., 1996) whilst, in airway epithelia, guanine nucleotide-binding proteins seem to allow apical $P2Y_2$ to exert direct control over ion channel activity (Stutts *et al*., 1992; 1994; Schwiebert *et al*., 1995).

Basolateral isoprenaline evoked a sustained rise in I_{SC} that was essentially abolished by amiloride (see also Rao & Cott, 1991; Ito *et al*., 1997) which accords with the view that basolateral β_2 adrenoceptors allow catecholamines to stimulate Na^+ absorption (Olver & Strang, 1974; Walters & Olver, 1978; Brown *et al*., 1983; Olver *et al*., 1986; O'Brodovich, 1991; Tohda *et al*., 1994; Matthay *et al*., 1996; Barker, 1997; Ito *et al*., 1997; Marunaka *et al*., 1999). Whilst there is no readily apparent reason why FDLE cells should have evolved an entirely separate apical control system to allow nucleotides to oppose this action, apical ATP clearly reversed this effect of isoprenaline. Conversely, following stimulation with ATP, isoprenaline could restore I_{SC} to its basal level. Our data thus establish a novel view of epithelial physiology in which basolateral β_2 adrenoceptors and apical $P2Y_2$ receptors allow mutually antagonistic control over Na^+ transport.

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