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$P2Y_2$-receptor-mediated activation of a contralateral, lanthanide-sensitive calcium entry pathway in the human airway epithelium

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1 Receptor-mediated calcium entry (RMCE) was examined in well-differentiated cultures of normal human bronchial epithelial cells (HBECs). Changes in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) were quantified using fluorescence ratio imaging of Fura-2-loaded cells during perfusion with Ca^{2+} mobilizing agonists.

2 Initial studies revealed an agonist potency of ATP = uridine triphosphate (UTP) > ADP = uridine diphosphate, consistent with purinergic activation of an apical $P2Y_2$-receptor mediating the increase in $[Ca^{2+}]_i$ in HBECs.

3 Apical UTP (30 μM) induced a sustained period of elevated $[Ca^{2+}]_i$ between 300 and 600 s following agonist stimulation that extracellular Ca^{2+} free studies indicated was dominated by Ca^{2+} influx.

4 RMCE was inhibited by 100 nM La^{3+} (83 ± 3%) or Gd^{3+} (95 ± 7%) ($P<0.005$, $n=4$–11) and was partially attenuated by Ni^{2+} (1 mM) (58.7 ± 5.0%, $P<0.005$, $n=9$).

5 RMCE was also partially sensitive (< 25% inhibition, $P<0.01$) to the cation channel blockers SKF96365 (30 μM) and econazole (30 μM), but was insensitive to both verapamil (1 μM) and ruthenium red (10 μM).

6 Using either a sided Ca^{2+} readdition protocol or unilateral La^{3+}, established that the RMCE pathway was located exclusively on the basolateral membrane.

7 The pharmacological sensitivity of the $P2Y_2$-receptor activated Ca^{2+} entry pathway in the human airway epithelium is inconsistent with the established profile of *TRP* channel families and is therefore likely to be of an as-yet uncharacterized molecular identity.

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Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; AUC, area under the curve; $AUC_{300-600}$, area under the curve between 300 and 600 s after UTP stimulation; BEGM, bronchial epithelial growth media; $[Ca^{2+}]_i$, intracellular free calcium; CPA, cyclopiazonic acid; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol-*O*,*O*′-bis(2-aminoethyl)-*N*,*N*,*N*′^*N*′-tetra-acetic acid; ER, endoplasmic reticulum; FU, 340 : 380 ratio fluorescence units; HBEC, human bronchial epithelial cell; HEPES, *N*-(hydroxyethyl)piperazine-*N*′-(2-ethanesulphonic acid); HBSS, Hanks' balanced salt solution; RMCE, receptor-mediated calcium entry; SKF96365, 1-[-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenyl]-1H-imidazole hydrochloride; *TRP*, transient receptor potential; UTP, uridine triphosphate

Introduction

Changes in the levels of intracellular free calcium $[Ca^{2+}]_i$ regulate a multitude of biological processes in both excitable and nonexcitable cells (Clapham, 1995). These signals can arise from either the release of Ca^{2+} from intracellular stores or the influx of Ca^{2+} through the plasma membrane. The emptying of stored Ca^{2+} can activate plasma membrane Ca^{2+} channels resulting in the influx of extracellular Ca^{2+}. This process is commonly known as 'Capacitative Calcium Entry' (CCE), or 'Store-operated calcium entry' (Putney & McKay, 1999). CCE serves to both refill depleted Ca^{2+} stores in the ER and to potentially prolong Ca^{2+}-dependent signalling. Alternatively, receptor stimulation can directly open plasma membrane Ca^{2+} channels without necessarily depleting intracellular Ca^{2+} stores, a process termed 'Receptor-Operated Calcium Entry' (Barritt, 1999).

Changes in $[Ca^{2+}]_i$ have been demonstrated to regulate several functions of the airway epithelium. Mucus secretion (Davis *et al*., 1992; Conway *et al*., 2003), surfactant secretion (Haller *et al*., 1998), ciliary beat frequency (Lansley *et al*., 1992) and ion transport mechanisms (Ribeiro *et al*., 2001) are all modulated by a variety of stimuli that elevate $[Ca^{2+}]_i$. The nucleotide triphosphates ATP and UTP are present in the airway surface liquid that bathes the mucosal (apical) surface of the airway epithelium (Donaldson *et al*., 2000; Lazarowski *et al*., 2000) and have emerged as key regulators of many of these processes through the activation of apical P2Y-receptors (Ribeiro *et al*., 2001). $P2Y_2$-receptor agonists are currently

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being tested in the clinic to enhance mucociliary clearance in cystic fibrosis (Kellerman, 2002).

Recent studies have investigated the Ca^{2+} influx pathways induced by purinergic agonists in the respiratory epithelium (Paradiso *et al*., 1995; Kerstan *et al*., 1999; Braiman and Priel, 2001; Zsembery *et al*., 2003). The reports are however inconsistent regarding the polarity of the RMCE pathway and to date there has been no description of the pharmacological sensitivity of Ca^{2+}-influx. The aims of the present study were therefore to utilize well-differentiated cultures of normal human bronchial epithelial cells (HBECs) to investigate: (1) the nature of the dominant purinergic receptor responsible for elevations in $[Ca^{2+}]_i$, (2) whether purinergic stimulation could induce RMCE, (3) the pharmacological sensitivity of the RMCE pathway, and (4) to determine the polarity of the pathway(s) using both Ca^{2+}-readdition and pharmacological approaches.

Methods

Cell culture

Nontransformed, primary HBECs (Biowhittaker, U.K.) (passage 1) were cultured as previously described (Danahay *et al*., 2002). At passage 3 (following expansion and freezing), cells were seeded (8.25×10^5 cells/insert) onto polycarbonate Snapwell ™ inserts (Costar, U.K.). Cells were maintained submerged for the first 7 days in culture after which time the apical media was removed and the cells exposed to an air–liquid interface for the remainder of the culture period. Cells were used between days 14 and 21 after establishment of the air–liquid interface. At all stages of culture, cells were maintained at 37°C in 5% CO_2 in an air incubator. HBECs from three donors were used for these studies. These culture conditions provide a well-differentiated epithelium composed primarily of ciliated, goblet and basal cells (Atherton *et al*., 2003) with an ion transport phenotype consistent with the native epithelium (Danahay *et al*., 2002). Although not specifically measured during the present studies, these cultures typically develop a transepithelial resistance of 400–1000 $\Omega.\,cm^2$.

Measurement of intracellular free calcium concentration $[Ca^{2+}]_i$

The apical surface of the HBECs was rinsed 3 times with warmed (37°C) HBSS (containing Ca^{2+}, Mg^{2+}, 20 mM HEPES, pH 7.4) (normal HBSS) to remove any accumulated mucus. Cells were then loaded (apical side only) with 5 μM Fura-2-AM in 200 μl of normal HBSS (containing pluronic acid 0.2%) for 60 min (37°C). The Fura-2-AM loading solution was then aspirated and the apical surface was again rinsed 3 times with normal HBSS. At this time, the culture media bathing the basolateral surface was replaced with 2 ml normal HBSS. Cells were then incubated for a further 30 min (37°C) and were placed into a custom-made, plastic chamber with normal HBSS bathing both apical and basolateral membranes (room temperature). The chamber enabled independent perfusion of both apical and basolateral surfaces with test solutions. The chamber was mounted on an upright Leica DMLM microscope and cells were observed with a $\times 20$ fluid immersion lens. Emitted fluorescence was observed after excitation was alternated at 340 and 380 nM by a 75 W xenon lamp linked to a Delta Ram illuminator (Photon Technology International (PTI) U.K.). Images of emitted fluorescence above 510 nM were recorded by an intensified CCD camera (PTI) and displayed on a monitor. The ratio of the emitted fluorescence following excitation at 340 and 380 nM was used as a surrogate measure of $[Ca^{2+}]_i$. The imaging system was under the control of ImageMaster software (PTI). At the completion of each experiment, background fluorescence was quantified following permeabilization with ionomycin ($\sim 50\,\mu$M) and quenching with $MnCl_2$.

Experimental protocols

At the start of all studies, normal HBSS was perfused over both apical and basolateral surfaces (2.5 ml min^{-1}) to establish a stable $[Ca^{2+}]_i$ baseline. For concentration–response experiments, cells were apically perfused with normal HBSS while the basolateral path was static. Following a 300 s stabilization period, normal HBSS solution was switched to the agonist-containing solution for 30 s, before reverting back to agonist-free normal HBSS. Pilot studies indicated that a 30 s perfusion was sufficient for the change in 340 : 380 ratio to reach the peak of the response. Perfusion with agonist-free normal HBSS continued until the 340 : 380 ratio had returned to baseline at which time the solutions were switched to deliver the next concentration of agonist. For experiments designed to test for UTP 'leak' from apical to basolateral membranes, apyrase (10 U ml^{-1}) was added to the basolateral perfusion solution. For low Ca^{2+} experiments, normal HBSS was exchanged for an HBSS (containing 100 μM Ca^{2+}, 1 mM Mg^{2+}, 200 μM EGTA, pH 7.4) (nominal Ca^{2+} solution) and was perfused over the appropriate epithelial surface(s). In the studies indicated, Ca^{2+} (1 mM) was added back to the nominal Ca^{2+} solution.

For pharmacological profiling of the RMCE pathway sensitivity, HBECs were perfused both apically and basolaterally with normal HBSS (containing vehicle or test inhibitor) for 300 s. The apical perfusion path was then switched to contain UTP (30 μM) (in the continued presence of vehicle or inhibitor). After 60 s of UTP perfusion apical and basolateral perfusion paths were stopped. Data acquisition was continued for a further 540 s resulting in a total UTP exposure of 600 s. In the La^{3+} and Gd^{3+} studies, normal HBSS was replaced with a phosphate-free salt solution containing (in mM): 140 NaCl, 5.4 KCl, 4.2 $NaHCO_3$, 5.6 glucose, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 20 HEPES (pH 7.4).

Expression of results and statistical analysis

Control inserts were run alongside all experiments for paired comparisons to be made owing to the potential day-to-day, interbatch and interdonor variability of responsiveness. Data are expressed as mean ($\pm$s.e.m.) absolute changes in the peak 340 : 380 ratio fluorescence units (FU) or as area under the curve for the 300–600 s period after UTP stimulation ($AUC_{300-600}$). Data are presented as FU rather than absolute values of $[Ca^{2+}]_i$ because of the difficulties involved in ensuring a robust and stable permeabilization of the HBECs that would be required to accurately calibrate the system. The

entire field that was visualized by the microscope was used as the region of interest for all calculations. A Student's *t*-test, with Bonferonni correction for multiple comparisons, was used to compare between control and test groups with statistical significance assumed when $P<0.05$.

Reagents

Unless otherwise stated, cell culture reagents and HBSS solutions were purchased from Life Technologies (U.K.). Fura-2-AM and pluronic acid were purchased from Molecular Probes (U.S.A.). All other reagents were purchased from Sigma (U.K.) unless otherwise stated.

Results

Apical UTP activates P2Y$_2$-receptors

Concentration–response studies using apical perfusions of nucleotide di- and triphosphates revealed a rank-order agonist potency profile for the peak increase in $[Ca^{2+}]_i$ of (mean $pEC_{50} \pm$ s.e.m.): ATP (5.54 ± 0.13) = UTP (5.72 ± 0.06) > ADP (3.94 ± 0.09) = UDP (3.95 ± 0.11) (Figure 1a), indicative of a P2Y$_2$-receptor-mediated response (Barnard *et al*., 1994). It should be noted that the nucleotide diphosphate solutions were not hexokinase treated to ensure depletion of any potential triphosphate contamination. It cannot therefore be excluded that the responses observed to UDP and ADP are as a result of a 1% contamination with UTP or ATP, respectively.

Apical P2Y$_2$-receptor activation stimulates Ca^{2+} influx

In normal HBSS buffer, apical UTP (30 μM) induced a rapid rise in $[Ca^{2+}]_i$ peaking at 1.45 ± 0.10 FU ($n=6$) that remained elevated above the starting baseline for the duration of the study (600 s following stimulation). When performed in a nominal Ca^{2+} solution, the peak increase was unchanged (1.42 ± 0.10 FU; $n=6$; $P>0.05$). However, under nominal Ca^{2+} conditions the increase in $[Ca^{2+}]_i$ had returned to baseline levels within 300 s of stimulation (Figure 1b). The period between 300 and 600 s after apical UTP stimulation was therefore considered to be dominated by RMCE ($AUC_{300-600}$).

In normal Ca^{2+} solution, UTP (30 μM) added to the basolateral perfusion solution induced an increase in $[Ca^{2+}]_i$ that peaked at 0.06 ± 0.01 FU ($n=5$) in contrast to the paired apical control which peaked at 1.22 ± 0.07 FU ($n=5$) (Figure 1c). The area under the curve for the basolateral UTP response accounted for $<5\%$ of the equivalent response to apical UTP in paired cells. In the presence of basolateral apyrase (10 U ml^{-1}), the $AUC_{300-600}$ in response to apical UTP (94.2 ± 9.5) was not different from the paired apyrase-free control (94.9 ± 5.3; $P>0.05$). This concentration of apyrase abolished the response to apical UTP (data not shown). These observations indicate that the changes in $[Ca^{2+}]_i$ observed following apical stimulation with UTP are indeed mediated *via* a direct effect at this membrane, rather than as a consequence of any putative paracellular leakage of exogenous UTP to enable basolateral membrane stimulation.

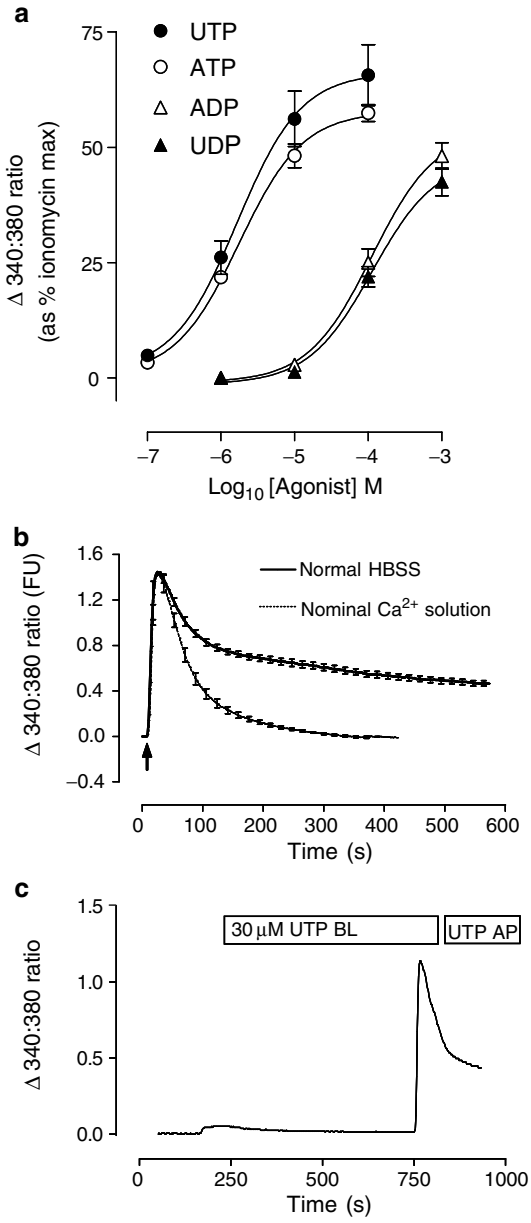

Figure 1 An apical P2Y$_2$-receptor mediates UTP-stimulated increase in $[Ca^{2+}]_i$ in HBECs. Potency order for purine nucleotide-evoked increase in $[Ca^{2+}]_i$ in HBECs (a). Each data point represents a mean ($\pm$s.e.m.) Δ 340 : 380 ratio normalized to the response achieved with a supramaximal concentration of ionomycin ($n=6$–15/group). Mean ($\pm$s.e.m.) kinetic data (b) illustrating the responses to UTP (30 μM) in either normal HBSS or nominal Ca^{2+} solution. Following a 300 s stabilization period before UTP-stimulation the peak increases in 340 : 380 ratio were not different between the two groups. In contrast, the 340 : 380 ratio had returned to baseline levels by 300 s after stimulation in the nominal Ca^{2+} solution while remaining elevated under normal Ca^{2+} conditions ($n=6$/group). Sample raw data trace illustrating the effect of basolateral UTP (30 μM) on the 340 : 380 ratio (c). Apical UTP (30 μM) was added at the end of the experiment to illustrate the responsiveness of the cells.

Pharmacological sensitivity of the RMCE in HBECs

The lanthanide cations, La^{3+} (Figure 2) and Gd^{3+} (Figure 3) blocked the Ca^{2+} influx response in UTP-stimulated HBECs in a concentration-dependent manner. The peak responses

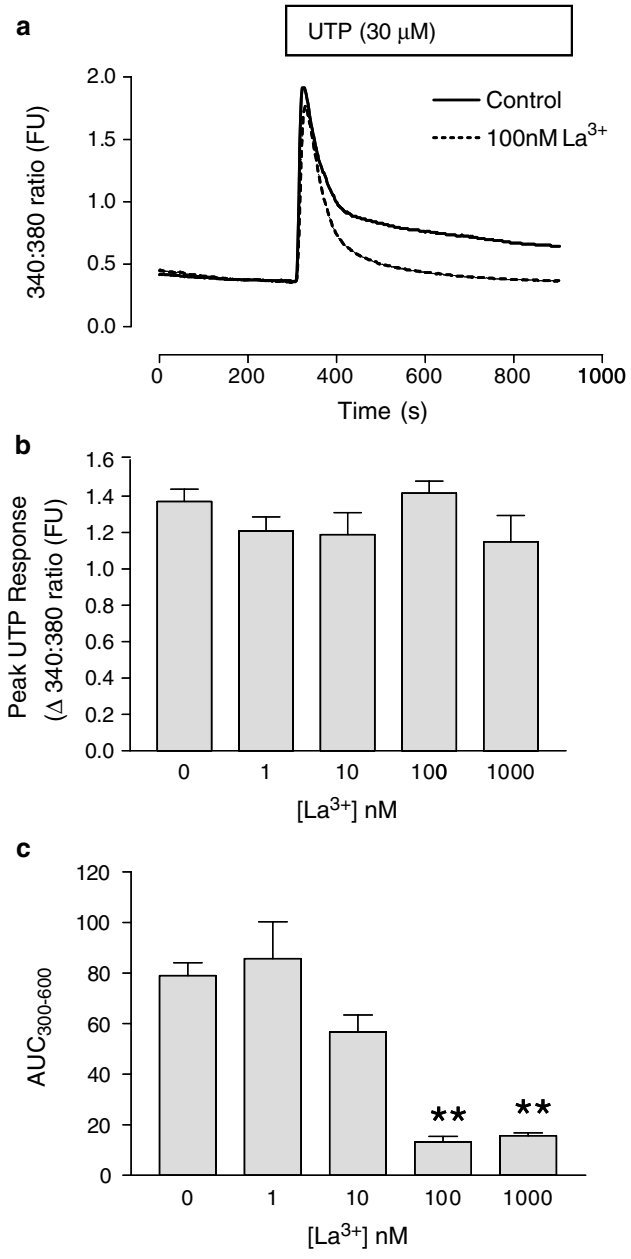

Figure 2 Sample raw data traces (a) illustrating the effect of La^{3+} (100 nM, apical and basolateral) on the profile of the apical UTP- (30 μM) stimulated elevation of $[Ca^{2+}]_i$ (absolute 340 : 380 ratio; FU). La^{3+} was without effect on the initial peak increase in $[Ca^{2+}]_i$ (b) but significantly attenuated the $AUC_{300-600}$ (c). Mean ($\pm$s.e.m.) data are shown ($n=4$–11/group). **Indicates $P<0.005$.

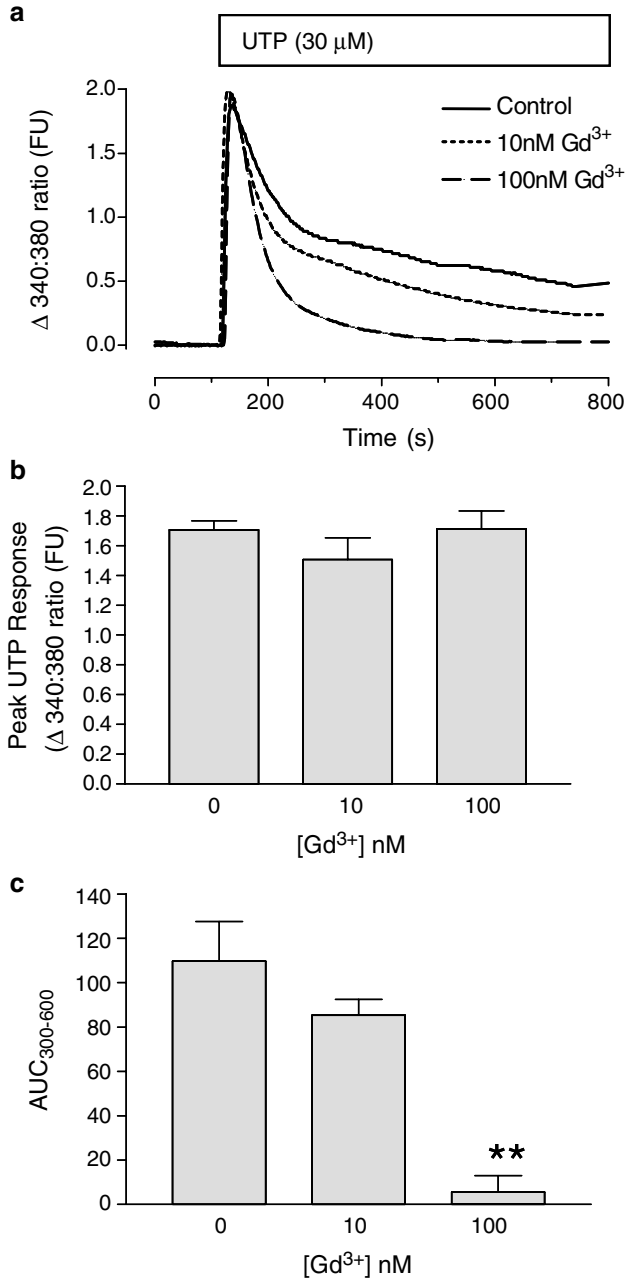

Figure 3 Sample raw data traces (a) illustrating the effect of Gd^{3+} (10 and 100 nM, apical and basolateral) on the profile of the apical UTP- (30 μM) stimulated elevation of $[Ca^{2+}]_i$ (Δ 340 : 380 ratio; FU). Gd^{3+} was without effect on the initial peak increase in $[Ca^{2+}]_i$ (b) but significantly attenuated the $AUC_{300-600}$ following stimulation with UTP (c). Mean ($\pm$s.e.m.) data are shown ($n=4$–11/group). **Indicates $P<0.005$.

to UTP were unaffected by the presence of either La^{3+} (1–100 nM) (Figure 2b) or Gd^{3+} (10–100 nM) (Figure 3b) indicating that Ca^{2+} release from intracellular stores was unaffected by the trivalent cations. In contrast, the lanthanides significantly attenuated the UTP-stimulated $AUC_{300-600}$ (Ca^{2+} influx) (Figures 2c and 3c). At 100 nM, La^{3+} and Gd^{3+} significantly inhibited $AUC_{300-600}$ by 83.2$\pm$2.8% ($P<0.005$; $n=4$) and 94.5$\pm$7.5% ($P<0.005$; $n=4$), respectively.

The non-selective cation channel blockers: Ni^{2+}, SKF96365 and econazole, all partially inhibited the $AUC_{300-600}$ Ca^{2+} influx response while having no effect on the initial peak increase induced by UTP (Table 1). In contrast verapamil, at a supra-maximal concentration to that required to inhibit L-VOCC channels (Glossmann & Striessnig, 1990), was without effect on either the peak or the $AUC_{300-600}$ influx responses. Ruthenium red, at a concentration previously demonstrated to block vanilloid receptors *TRPV-1* and *TRPV-5* (Tominaga *et al*., 1998; Nilius *et al*., 2001), was also without effect on either phase of the UTP-stimulated Ca^{2+} response. It was impractical to use higher concentrations of ruthenium red because of interference with the Fura-2 fluorescence.

Table 1 Effects of cation channel blockers on UTP-induced $\Delta[Ca^{2+}]_i$

Compound	*Conc* (μM)	n	*Peak (% inhibition)*	*$AUC_{300-600}$ (% inhibition)*
Ni^{2+}	100	4	−1.3±13.1	−2.1±1.9
	300	4	−7.7±9.3	14.4±10.4
	1000	9	0.4±7.7	58.7±5.0**
Verapamil	1	4	−1.2±7.2	5.7±8.6
SKF96365	10	6	13.6±6.4	3.1±4.8
	30	5	9.6±6.8	20.6±2.1**
Econazole	3	4	−1.7±2.2	−13.7±6.2
	10	5	−3.6±4.5	9.6±4.6
	30	11	−5.1±5.2	26.7±3.9**
Ruthenium red	10	7	−3.6±5.5	3.0±6.1

Data represent the mean (±s.e.m.) % Inhibition of the initial peak increase in $[Ca^{2+}]_i$ (Peak) and the phase of RMCE ($AUC_{300-600}$) following stimulation of HBECs with UTP. **Indicates $P<0.005$ (test compound vs paired vehicle control).

2-Aminoethoxydiphenyl borate (2-APB) has been used as an inhibitor of Ca^{2+} mobilisation *via* the blockade of IP_3 receptors (Maruyama *et al*., 1997). At 50 μM the $AUC_{300-600}$ was significantly inhibited by 92.5±3.6% ($P<0.005$; $n=5$) (Figure 4b); however, the peak response was also significantly reduced by 17.0±3.5% ($P<0.05$) (Figure 4a). A similar, although more pronounced profile was observed for 2-APB (75 μM). 2-APB (10 μM) was without significant effect on either component of the response.

Apical $P2Y_2$-receptor stimulation activates a basolateral RMCE pathway in HBECs

Ca^{2+} readdition Apical UTP (30 μM) stimulation of HBECs in nominal Ca^{2+} solution (apical and basolateral) resulted in a rapid Ca^{2+} transient which returned to baseline values within 300–400 s poststimulation. Subsequent switching of the apical perfusion path to normal HBSS (1 mM Ca^{2+}) had no effect on $[Ca^{2+}]_i$ (Figure 5a). In contrast, switching the basolateral perfusion solution to normal HBSS (1 mM Ca^{2+}) resulted in a rise in $[Ca^{2+}]_i$. In the paired control experiment (Figure 5b), an identical UTP-stimulation was performed in nominal Ca^{2+} solution but this time the order of Ca^{2+} readdition was reversed. Again, the basolateral readdition of Ca^{2+} resulted in an increase in $[Ca^{2+}]_i$ while subsequent apical readdition was without effect. The magnitude of the peak Ca^{2+} responses that occurred in response to the apical and then basolateral readdition of 1 mM Ca^{2+} are illustrated in Figure 5c.

Basolateral or apical La^{3+} on the RMCE pathway Pretreatment of the basolateral surface of HBECs with La^{3+} (100 nM, 300 s) in normal HBSS followed by stimulation of the apical surface with UTP (30 μM) significantly attenuated the $AUC_{300-600}$ by 84.7±7.4% ($P<0.005$; $n=6$) compared to the vehicle control (Figure 6). Conversely, pretreatment of the apical surface with La^{3+} (100 nM, 300 s) was without significant effect on the UTP-stimulated $AUC_{300-600}$.

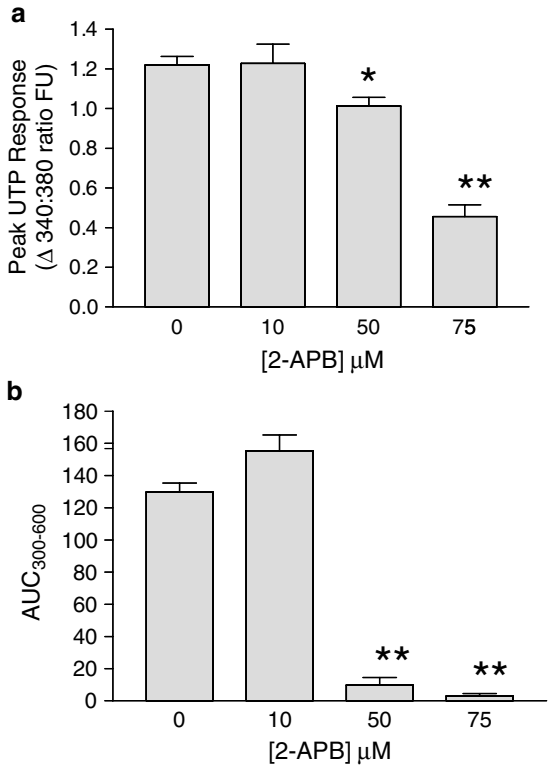

Figure 4 2-APB significantly attenuated both the initial peak increase in $[Ca^{2+}]_i$ (Δ 340 : 380 ratio; FU) (a) and the $AUC_{300-600}$ following stimulation with UTP (b). Mean (±s.e.m.) data are shown ($n=5$–15/group). *Indicates $P<0.05$. **Indicates $P<0.005$.

Discussion and conclusions

In the present study, we have examined well-differentiated cultures of normal human bronchial epithelial cells to establish: (1) that an apical $P2Y_2$-receptor is responsible for nucleotide triphosphate stimulated increases in $[Ca^{2+}]_i$, (2) apical UTP stimulates a biphasic elevation of $[Ca^{2+}]_i$, that from 300 s after stimulation is dominated by RMCE ($AUC_{300-600}$), (3) a pharmacological profile inhibition of RMCE that is characterised by high sensitivity to the lanthanides La^{3+} and Gd^{3+}, and (4) that this RMCE pathway is located exclusively on the basolateral membrane.

In view of recent literature reports, that have used a variety of airway epithelial preparations to study purinergic-receptor-mediated events (Communi *et al*., 1999; Ribeiro *et al*., 2001; Ito *et al*., 2003; Zsembery *et al*., 2003), it was important to confirm that an apical $P2Y_2$-receptor was responsible for the nucleotide triphosphate-stimulated increase in $[Ca^{2+}]_i$ in our HBEC cultures (Figure 1a). The inclusion of apyrase in the basolateral solution, and the relative lack of responsiveness to UTP applied to the basolateral membrane, confirmed that the effect of UTP was specific to the apical membrane and that any putative paracellular leakage of exogenous UTP had no significant impact on the increase in $[Ca^{2+}]_i$. The removal of extracellular Ca^{2+} confirmed that following apical stimulation with UTP, there followed a sustained phase of Ca^{2+} entry (Figure 1b) that was quantified as the $AUC_{300-600}$.

Studies were then undertaken to establish the pharmacological sensitivity of the conductance(s) responsible for the passage of Ca^{2+} across the plasma membrane following apical

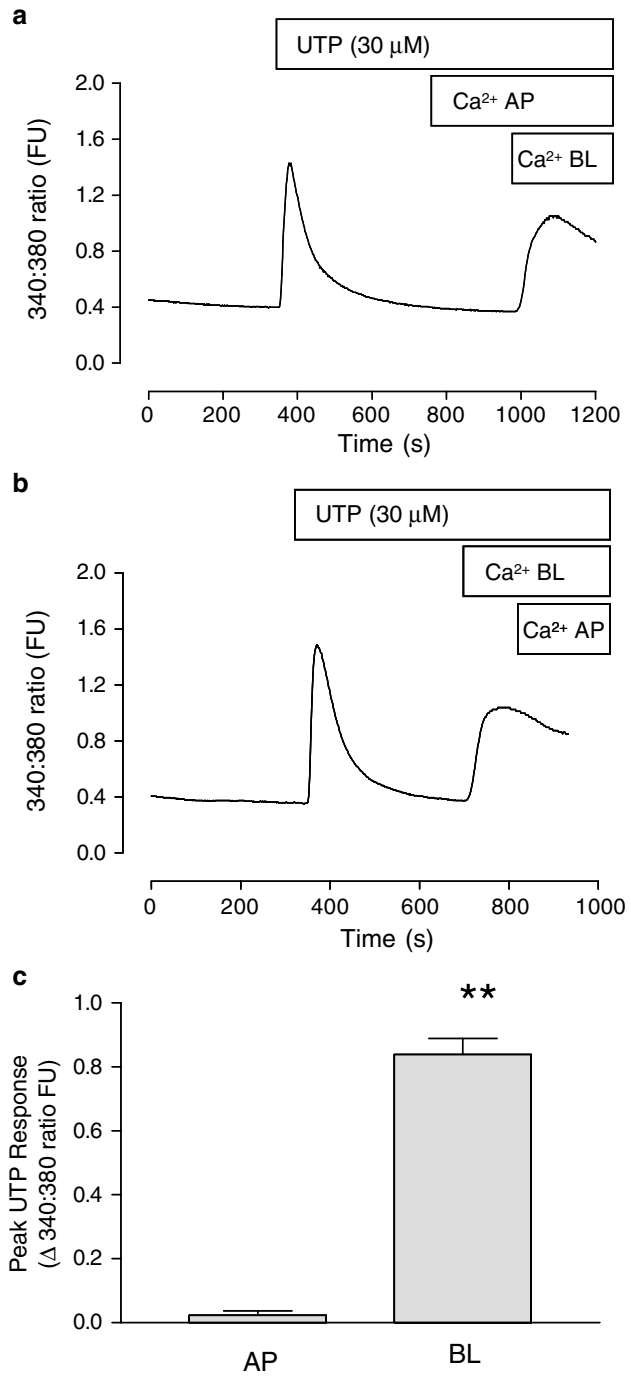

Figure 5 Sample raw data traces illustrating the effects of readdition of Ca^{2+} (normal HBSS) to the apical (AP) and then basolateral (BL) (a) or basolateral and then apical (b) membranes of HBECs previously stimulated with UTP (30 μM) in nominal Ca^{2+} solution. Mean ($\pm$s.e.m.) data showing peak responses Ca^{2+} readdition apical then basolateral (Δ 340 : 380 ratio; FU) (c). **Indicates significant difference ($P<0.005$) between AP and BL readdition ($n=6$/group).

$P2Y_2$-receptor activation. The effects of test compounds on $AUC_{300-600}$ was preferred to using a Ca^{2+} readdition protocol with subsequent addition of blocker (Clementi *et al*., 1992) as at no time was it necessary to expose the cells to a Ca^{2+}-free solution that might influence the nature of a subsequent Ca^{2+} entry pathway.

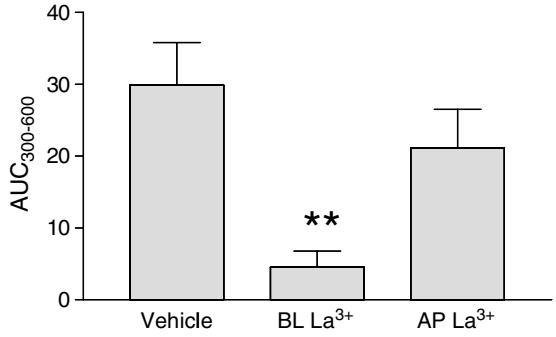

Figure 6 La^{3+} (100 nM) when included in the basolateral (BL) perfusion solution significantly attenuated the $AUC_{300-600}$ following stimulation with UTP. Apical (AP) La^{3+} was without significant effect. Mean data ($\pm$s.e.m.) are shown. **Indicates significant difference ($P<0.005$) between vehicle control and basolateral (BL) La^{3+} ($n=6$/group).

Verapamil, a potent blocker of voltage-operated Ca^{2+} channels (Glossmann & Striessnig, 1990) was without effect on either the peak increase in $[Ca^{2+}]_i$ or the RMCE phase indicating no role for this class of conductance in the $P2Y_2$-receptor stimulated Ca^{2+}-influx. The partial attenuation of the RMCE phase by the nonselective cation channel blockers: Ni^{2+} (1 mM), SKF96365 (30 μM) and econazole (30 μM) and the lack of effect of ruthenium red are consistent with a putative blocking effect on members of the *TRPC* (Li *et al*., 2002) rather than *TRPV* families of transient receptor potential cation conductances (Tominaga *et al*., 1998; Nilius *et al*., 2001; Peng *et al*., 2003). The effect of 2-APB was also consistent with inhibition of a CCE pathway, although at the concentrations used there were also significant effects on the release of stored Ca^{2+} potentially as a consequence of its well-established activity as an IP_3-receptor antagonist (Maruyama *et al*., 1997). The intriguing aspect of the inhibitor profile was the low (100 nM) concentration of La^{3+} or Gd^{3+} that effectively inhibited the RMCE pathway. These concentrations are substantially lower than those widely used to demonstrate effects on *TRPC* and *TRPV* family members (5–50 μM). Interestingly, Gd^{3+} has been demonstrated to block CCE in both cultured (Broad *et al*., 1999) and primary vascular smooth muscle cells (Fellner & Arendshorst, 2002) at concentrations similar to those used to inhibit Ca^{2+} influx in the present study. The identity of the conductance(s) involved in these smooth muscle studies are unknown. Likewise, lanthanides inhibit CCE in both HL-60 cells and primary human neutrophils with IC_{50} values between 20 and 250 nM (P. Bahra *et al.,* unpublished observations). This profile of sensitivity is consistent with a $P2Y_2$-receptor stimulated capacitative Ca^{2+} entry pathway (i.e. as a result of store depletion) although inconsistent with a role for the *TRP*-family of cation conductances. It should however be considered that the published pharmacology of the *TRP*-channels has been elucidated in heterologous expression systems rather than in native tissues, a factor that could alter the profile of blocker sensitivities.

The observation that the apical $P2Y_2$-receptor stimulated RMCE pathway is exclusively localized to the basolateral membrane was confirmed using both the classical Ca^{2+} readdition technique (Clementi *et al*., 1992) and by utilizing La^{3+} as a potent blocker of the HBEC Ca^{2+} entry

conductance. The basolateral location of the RMCE pathway and that it is contralateral to the agonist-stimulated membrane, are in agreement with observations made using the human bronchial epithelial cell line 16HBE14o$^-$ (Kerstan *et al*., 1999). However, in contrast, a RMCE pathway ipsilateral to the ATP-stimulated membrane has been described in both human nasal (Paradiso *et al*., 1995; Braiman and Priel, 2001) and tracheobronchial epithelial cultures (Ribeiro *et al*., 2003). As discussed above, the apyrase studies confirmed that the stimulation of the RMCE was not due to the paracellular 'leak' of exogenous UTP from the apical to basolateral membranes (Figure 1c). Furthermore, the response to direct stimulation of the basolateral membrane with UTP (30 μM) could not account for the magnitude of the observed phase of Ca^{2+}-influx. This disparity between our observations and the study by Ribeiro *et al*. (2003) that used similar HBEC methodology to our own, are therefore difficult to reconcile. A number of studies support the concept that a P2Y-receptor-mediated stimulus can indeed traverse airway epithelial cells to induce functional effects at the contralateral membrane in terms of both basolateral to apical signal transduction (Davis *et al*., 1992; Paradiso *et al*., 1995) and apical to basolateral transduction (Devor & Pilewski, 1999). Furthermore, it has been demonstrated that a RMCE pathway on the basolateral membrane of pancreatic acinar cells is able to refill ER in the apical pole of the cell (Petersen *et al*., 1999).

The functional relevance of Ca^{2+}-influx into the airway epithelium, beyond the refilling of depleted stores, remains largely unknown. Certainly, purinergic stimulation of the airway epithelium has been demonstrated to elevate $[Ca^{2+}]_i$, and to regulate a variety of functions including mucin and surfactant secretion, ciliary beat frequency and anion secretion, all of which influence the process of mucociliary clearance. RMCE has been demonstrated to regulate sustained anion secretion in 16HBE14o$^-$ cells (Kerstan *et al*., 1999). Recently, Bertrand *et al*. (2004) demonstrated that degranulation of the colonic goblet cell line HT29 was also partially dependent upon a rapid phase of La^{3+}- and niflumic acid-sensitive Ca^{2+} influx following stimulation with ATP.

In conclusion, we have established that the apical stimulation of $P2Y_2$-receptors results in the sustained activation of a basolateral RMCE pathway. This pathway is partially sensitive to blockers of the known *TRPC* cation conductance family but also to low (10–100 nM) concentrations of the trivalent cations, La^{3+} and Gd^{3+}. The molecular identity of the conductance(s) responsible for the pathway and its functional relevance to the airway epithelium, beyond that of simply refilling ER Ca^{2+} stores, remains to be established. In view of the potential utility of $P2Y_2$-receptor agonists as therapeutics to enhance mucociliary clearance, modulators of the RMCE pathway may represent novel drug targets.

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