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SPECIAL REPORT

Culture substrate-specific expression of $P2Y_2$ receptors in distal lung epithelial cells isolated from foetal rats

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ATP and UTP did not evoke $[Ca^{2+}]_i$ signals in rat foetal lung epithelial cells grown on glass but elicited clear responses in cells grown into functionally polarised epithelia on permeable supports. Moreover, $P2Y_2$ receptor mRNA could not be detected in cells on glass by the polymerase chain reaction but this mRNA species was clearly expressed by polarised cells. $P2Y_2$ receptor expression thus appears to be a feature of the polarised phenotype.

Keywords: Epithelial polarity; polymerase chain reaction; apical membrane; nucleotide receptors; epithelial cells; lung; apical membrane

Introduction $P2Y_2$ receptors, which are equally sensitive to ATP and uridine 5′ triphosphate (UTP) but insensitive to nucleotide diphosphates (see Nicholas *et al*., 1996), are expressed by rat type II pneumocytes (Rice *et al*., 1995). Whilst their physiological role is unknown, these receptors could allow surfactant secretion and alveolar ion transport to be controlled by nucleotides (Rice *et al*., 1995; Clunes & Kemp, 1996). These processes are subject to rapid change as the foetal lung is prepared for air breathing at birth and so the aim of the present study was to establish if $P2Y_2$ receptors were also present in the undifferentiated, distal epithelium of the foetal lung.

Methods Foetuses removed from anaesthetised (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. The tissue was then chopped into pieces (< 0.5 mm), digested using 0.2% trypsin/0.012% DNAase (2 × 20 min, 37°C) followed by 0.1% collagenase/0.012% DNAase (15 min, 37°C, both in Dulbecco's modified Eagle's medium, DMEM), and the resultant digest incubated (37°C) 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, non-adherent cells were resuspended in DMEM containing 10% foetal bovine serum. Standard immunocytochemical analyses showed that > 95% of these cells expressed cytokeratin, confirming their epithelial lineage, whilst < 5% expressed vimentin, demonstrating negligible fibroblast contamination. Cells were plated (10^6 cells cm^{-2}) onto Transwell Clear membranes (Costar, High Wycomb, Bucks.), glass coverslips or glass Petri dishes, and non-viable cells removed, after 24 h, by gently washing each culture. Cells were then incubated for 24 h in serum-free PC-1 medium before being used in experiments. Transepithelial resistance and short circuit current (I_{SC}) were measured by mounting cells growing on Transwell membranes into Ussing chambers (e.g. Wilson *et al*., 1998). Changes in $[Ca^{2+}]_i$ were monitored by loading cells on coverslips or Transwell membranes with Fura-2 (e.g. Clunes & Kemp, 1996; Wilson *et al*., 1998). In these experiments, the superfusion system used allowed nucleotides to be delivered to the apical (i.e. upward facing) side of the cells on Transwell membranes. Total RNA was isolated using Tri-reagent (Sigma Chemical Co., Poole, Dorset) and the mRNA reverse-transcribed into cDNA using oligo dT primers and MMLV reverse transcriptase (Clontech, Palo Alto, CA, U.S.A). Aliquots of this cDNA were then used as templates for the polymerase chain reaction (PCR).

Results After 48 h in culture, cells on Transwell filters ($n = 23$) had formed coherent layers (transepithelial resistance: $1309 \pm 93\ \Omega.cm^2$) that spontaneously generated I_{SC} ($14.0 \pm 1.6\ \mu A\ cm^{-2}$). This basal current was reduced ($74.6 \pm 3.1\%$, $P < 0.05$, Student's paired t test), although not abolished, by apical amiloride (10 μM) and so, at least in part, it can be attributed to electrogenic sodium transport. Apical ATP (100 μM) elicited a transient rise in I_{SC} followed by a decline to a value below baseline ($n = 3$). UTP (100 μM) evoked an essentially identical response ($n = 3$).

The transport process(es) underlying this basal current are thus subject to acute regulation *via* apical receptors sensitive to ATP and UTP. To explore the possibility that it may be $[Ca^{2+}]_i$-mobilising $P2Y_2$ receptors that permit this control (Rice *et al*., 1995; Nicholas *et al*., 1996), $[Ca^{2+}]_i$ was monitored in cells grown on coverslips. This is a standard, physiological approach. However, ATP (Figure 1a) and UTP ($n = 7$) failed to evoke discernible responses in this preparation although the cells in which nucleotides were tested subsequently showed a clear response to 20 μM 2,5′-di-(*tert*-butyl)-1,4,-benzohydroquinone (TBQ, rise in Fura-2 ratio: 0.28 ± 0.04). TBQ increases $[Ca^{2+}]_i$ *via* a receptor-independent mechanism (see Clunes & Kemp, 1996) and so this response confirms that $[Ca^{2+}]_i$ was being measured. In subsequent studies $[Ca^{2+}]_i$ was monitored in the functionally polarised cells on Transwell membranes. Apical ATP (Figure 1b) and UTP consistently increased $[Ca^{2+}]_i$ in these cells and the EC_{50} values were 3.0 ± 0.5 μM and 2.9 ± 0.4 μM respectively (Figure 1c). These nucleotides thus act with essentially identical efficacy and potency (Figure 1c) and experiments ($n = 7$) using a classical, cross desensitisation

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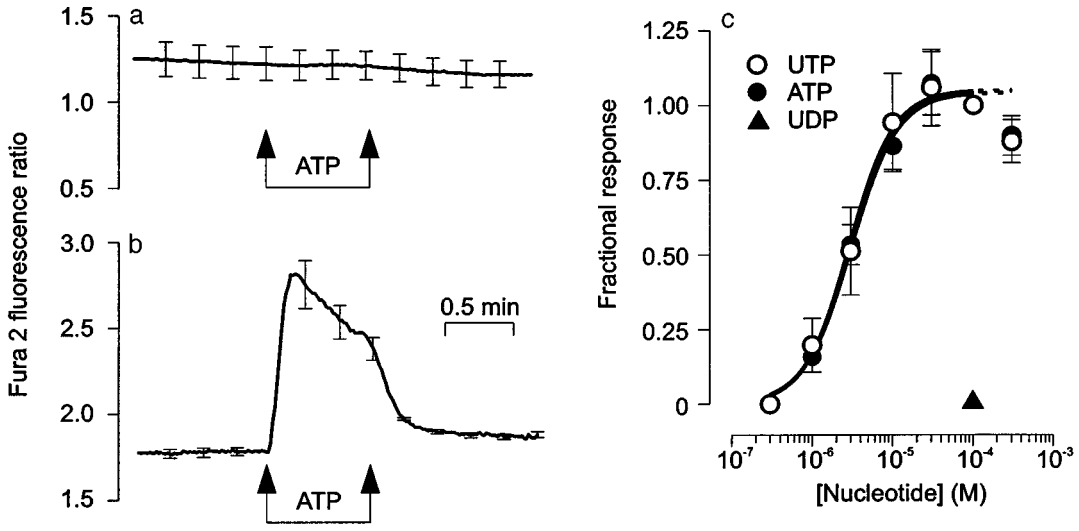

Figure 1 Fura-2 fluorescence ratios (mean ± standard error) recorded from lung epithelial cells plated onto glass coverslips (a, $n=7$) or Transwell clear filters (b, $n=4$). Cells were superfused ($\sim$3 ml min^{-1}) with HEPES-buffered saline (composition, in mM: NaCl, 100; KCl, 5, $MgCl_2$, 1; $CaCl_2$ 1; HEPES, 20, D-glucose, 10; pH adjusted to 7.4 with NaOH) and exposed to 10 μM ATP as indicated. (c) The peak increases Fura-2 fluorescence ratio evoked by a series of 40 s pulses of increasing nucleotide concentrations (0.3–300 μM) are plotted against the concentration of nucleotide used. Pulses were delivered to the cells at intervals of at least 5 min and each data point shows the mean ± standard error of 4–8 observations. EC_{50} values were estimated from sigmoid curves fitted to the experimental data using commercially available software (*Grafit 3*, Erithacus Software, Staines U.K.).

protocol suggested that they acted upon a common receptor population. UDP was ineffective (Figure 1c).

Apical nucleotides thus evoke $[Ca^{2+}]_i$ signals in cells grown on Transwell membranes, apparently by activating $P2Y_2$ receptors (Nicholas *et al*., 1996), but cells on glass appear insensitive to nucleotides. We therefore used a PCR-based assay to determine if this reflected differential expression of the $P2Y_2$ receptor gene. In these experiments, rat $P2Y_2$ primers (Rice *et al*., 1995) yielded a single product using cDNA derived from cells on Transwell membranes (Figure 2), and the product's size and nucleotide sequence confirmed that it originated from rat $P2Y_2$ receptor mRNA. However, no such product was obtained using cDNA derived from cells grown on glass but analogous experiments showed that $P2Y_2$ receptor mRNA was present in acutely isolated cells that had not been maintained in culture. Expression of the $P2Y_2$ receptor gene is thus maintained by cells grown into functionally polarised epithelia but not by cells simply grown on a glass substrate.

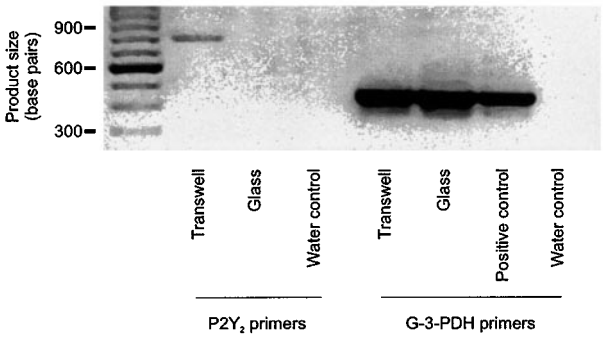

Figure 2 PCR products obtained using aliquots of cDNA corresponded to 180 ng of total RNA that was isolated either from cells grown on glass Petri dishes or Transwell membranes. PCR was continued for 35 denaturing/annealing/polymerisation cycles (94°C, 45 s/60°C, 45 s/72°C, 2 min) and the resultant products fractionated on 1% (w/v) agarose/ethidium bromide gels and visualised under ultraviolet light. $P2Y_2$ primers were designed to amplify a cDNA sequence specific to the rat $P2Y_2$ receptor gene sequence presented by Rice *et al.* (1995) (upstream primer: 5′-CTTCAACGAGGACTT-CAGTATGTGC-3′, corresponding to nucleotides 78–103; downstream primer: 5′-CATGTTGATGGCGTTGAGGGTGTGG-3′, corresponding to nucleotides 855–831) whilst the G-3-PDH primers, together with the appropriate, synthetic cDNA positive control were obtained from Clontech (Palo Alto, CA, U.S.A). The G-3-PDH gene is a 'housekeeping' gene expressed by all viable cells; the presence of the PCR product obtained using these primers thus confirms that the failure to generate a product using the $P2Y_2$ primers is not due to failure of the RNA isolation, reverse transcription or PCR procedures. Water control lanes serve to confirm that no product is obtained if the cDNA is omitted from the reaction mixture.

Discussion Whilst studies of non-epithelial cell types have established that $P2Y_2$ receptor gene expression is an acutely regulated event (Koshiba *et al*., 1997; Martin *et al*., 1997), the present data show that foetal distal lung epithelial (FDLE) cells must form functionally polarised layers if $P2Y_2$ receptor expression is to be retained *in vitro*. In these cells, $P2Y_2$ receptor expression thus appears to be associated intimately with the maintenance of a distinct, apical membrane. Recent data from an equine epithelial cell line are interesting in this context. These cells, in contrast to FDLE cells, expressed $P2Y_2$ receptors when grown on glass but, in polarised cultures, the receptors became confined to the apical membrane (Wilson *et al*., 1998). Most importantly, however, the apical membranes of these cultured epithelia contained at least one P2Y receptor subtype that was not present in cells that had been simply grown on glass. These unidentified receptors were activated by UTP and UDP but not by ATP (Wilson *et al*., 1998) and so, most probably, belong to the $P2Y_6$ subclass (Nicholas *et al*., 1996; Communi & Boeymans, 1997). It thus appears that the epithelial expression of at least two P2Y receptors subtypes is linked to the polarised phenotype (present study, Wilson *et al*., 1998) and these data establish a new and potentially important determinant of epithelial P2Y receptor expression.

In both systems (present study, Wilson *et al*., 1998), the receptors associated with polarisation are found in the apical membrane. Such apical receptors have been identified in many

previous studies (e.g. Schwiebert *et al*., 1995) but their physiological role remains enigmatic. It has, however, been suggested that such apical receptors might be activated by ATP released across the apical membranes of stimulated or mechanically traumatised epithelia, and so allow nucleotides released in this way to exert autocrine control over epithelial function (Schwiebert *et al*., 1995; Lazarowski *et al*., 1997). As $P2Y_2$ and $P2Y_6$ receptors now appear to be linked to the polarised phenotype (present study, Wilson *et al*., 1998), it is likely that these receptors are involved in this process. However, these receptors' unifying feature is their sensitivity to *pyrimidine* nucleotides rather than to ATP (Nicholas *et al*., 1996; Communi & Boeymans, 1997). It is now clear, however, that mechanical stimulation induces the release of sufficient UTP to allow autocrine activation of such receptors (Lazarowski *et al*., 1997). As epithelial polarisation appears to induce the expression of apical receptors sensitive to UTP and UDP (present study, Wilson *et al*., 1998) it is possible that these substances, as well as ATP (Schwiebert *et al*., 1995), may act as autocrine signalling molecules.

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