J. Membrane Biol. 170, 205–211 (1999)

The Journal of
Membrane
Biology

The Simultaneous Measurement of Epithelial Ion Transport and Intracellular Free Ca^{2+} in Cultured Equine Sweat Gland Secretory Epithelium

W.H. Ko[1], V.W.Y. Law[1], H.Y. Wong[1], S.M. Wilson[2]

[1]Department of Physiology, The Chinese University of Hong Kong, New Territories, Hong Kong, China
[2]Lung Membrane Transport Group, Tayside Institute of Child Health, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, UK

Received: 8 December 1998/Revised: 23 April 1999

Abstract. We explored the relationship between nucleotide-evoked changes in intracellular free calcium ($[Ca^{2+}]_i$) and anion secretion by measuring $[Ca^{2+}]_i$ and I_{SC} simultaneously in Fura-2-loaded, cultured equine sweat gland epithelia. Apical ATP, UTP or UDP elicited sustained increases in $[Ca^{2+}]_i$ that were initiated by the mobilization of cytoplasmic Ca^{2+} but maintained by Ca^{2+} influx. However, although these nucleotides also increased I_{SC}, this response was transient whereas the $[Ca^{2+}]_i$ signals were sustained. Experiments in which external Ca^{2+} was removed/replaced showed that Ca^{2+} entering nucleotide-stimulated cells elicited very little change in I_{SC}. Cross desensitization experiments showed that UTP-stimulated epithelia became insensitive to ATP but that UTP could increase both $[Ca^{2+}]_i$ and I_{SC} in ATP-stimulated cells by activating 'pyrimidinoceptors' essentially insensitive to ATP. Thapsigargin evoked a sustained rise in $[Ca^{2+}]_i$ that was accompanied by a maintained increase in I_{SC}. However, this increase in I_{SC} was dependent upon external Ca^{2+} and so the responses to nucleotides and thapsigargin have different properties. ATP increased I_{SC} in thapsigargin-treated cells without causing any rise in $[Ca^{2+}]_i$ while ionomycin increased both parameters. The data therefore show that apical P2Y receptors allow nucleotides to increase I_{SC} *via* two mechanisms, one of which appears to be $[Ca^{2+}]_i$-independent control of anion channels.

Key words: P2Y receptors — Apical membrane — Epithelial anion secretion — Ussing chamber — Stimulus-secretion coupling —Intracellular Ca^{2+}

Correspondence: S.M. Wilson

Introduction

P2Y receptors are found in the apical membranes of essentially all polarized epithelia where they allow nucleotides to increase intracellular free calcium ($[Ca^{2+}]_i$) and exert control over transepithelial ion transport processes. Although this signaling system's physiological role is unclear, it may form part of a widespread autocrine control mechanism [2, 4, 11, 12, 16, 17, 26, 30, 32, 33, 35, 38]. Moreover, it is possible that this pathway may be exploited to allow pharmacological correction of the ion transport defects that cause chest disease in cystic fibrosis, a potentially lethal genetic disease [12, 22]. Identifying the mechanisms that allow apical nucleotides to exert control over epithelial function has, therefore, become an important problem in modern, cellular physiology [12].

It has recently become clear that the epithelial expression of at least some P2Y receptor subtypes is associated specifically with the maintenance of a distinct, apical membrane [4, 10, 33]. However, many experimental approaches used to study epithelial cell signaling, such as fluorescent measurements of intracellular free calcium ($[Ca^{2+}]_i$), isotope efflux techniques and electrophysiological studies of single cells [*see* e.g., 15, 22, 32, 36, 40], are almost invariably applied to cells on coverslips or Petri dishes. As these conditions do not favor epithelial polarization, such experiments may well fail to reveal the means by which nucleotides control ion transport in polarized epithelia. Moreover, the electrometric techniques that are often used to monitor ion transport in polarized epithelia [*see* e.g., 31, 38] do not readily lend themselves to the detailed study of intracellular events. There is, therefore, a need for a technique that will allow intracellular signaling and transepithelial ion transport to be monitored in polarized cells. In the present study, we

therefore use a new technique, which allows these parameters to be measured concurrently [*see also* 18, 23], to explore the relationship between nucleotide-evoked $[Ca^{2+}]_i$ signals and ion transport in cultured equine sweat gland secretory epithelia.

Materials and Methods

Solutions and Chemicals

The culture medium was William's medium E containing L-glutamine (1 mM), penicillin (100 i.u. ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$), bovine insulin (5 $\mu g\ ml^{-1}$), epidermal growth factor (0.1 $\mu g\ ml^{-1}$), hydrocortisone (10 $ng\ ml^{-1}$), transferrin (5 $\mu g\ ml^{-1}$) and sodium selenite (5 $ng\ ml^{-1}$). The 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes)-buffered saline contained (in mM): NaCl, 120; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1; D-Glucose, 10; Hepes, 20; pH adjusted to 7.4 with NaOH. The membrane permeant, acetoxymethylester (AM) forms of Fura-2 and 1,2-bis-(2-aminophenoxy)-ethane-N,N,N′N′-tetraacetic acid (BAPTA) were from Molecular Probes (Eugene, OR) and Calbiochem (La Jolla, CA), respectively. Uridine diphosphate (UDP) was from Boehringer (Mannheim, Germany) but, before use, this nucleotide (10 mM) was incubated (1 hr at 37°C) in Hepes-buffered saline containing hexokinase (10 i.u. ml^{-1}, Boehringer, Mannheim, Germany) and 22 mM D-glucose in order to remove contaminating nucleotide triphosphates. The resulting solution was aliquoted and stored at −20°C. Uridine triphosphate (UTP) and adenosine triphosphate (ATP) were obtained from Pharmacia as ultrapure solutions. Independent analyses have confirmed that these nucleotides are essentially pure (R.C. Boucher, *personal communication*). Thapsigargin was from Calbiochem, diphenylamine-2-carboxillic acid (DPC) from Riedel-de-Haën Chemicals (Hannover, Germany), ionomycin, 4,4′-diisothiocyanateostilbene-2,2′-disulfonic acid (DIDS) and all other nucleotides and general laboratory reagents were from Sigma, and cell culture reagents were from Gibco Laboratories (New York).

Cell Culture

Experiments were undertaken using a spontaneously transformed cell line (E/92/3) derived from the secretory epithelium of the equine sweat gland [*see* 34]. These cells were maintained in serial culture using standard techniques. For experiments, cells were removed from culture flasks using trypsin/EDTA, washed by centrifugation/resuspension in fresh medium and aliquots of this suspension plated (2.2×10^5 cells cm^{-2}) onto permeable supports fabricated from Transwell-Col membranes (0.4 μm pore diameter, Costar, Cambridge, MA). The culture area was 0.1 cm^2. Membranes bearing cultured cells were floated on culture medium in Petri dishes and incubated for the 3 days; previous work showed that E/92/3 cells consistently become integrated into functionally polarized monolayers when maintained under such conditions [13]. These cultured epithelia were then loaded with Fura-2, a Ca^{2+} sensitive fluorescent dye, by incubation (45 min, 37°C) in medium containing 3 μM Fura-2-AM and 1.6 μM pluronic F127. Changes in $[Ca^{2+}]_i$ elicit corresponding changes in the Fura-2 fluorescent ratio recorded from cells loaded with this dye, which allows changes in $[Ca^{2+}]_i$ to be monitored using standard, microspectrofluorimetric techniques [7]. In the present study, the cultured epithelia took up very little Fura-2 under standard conditions and so inclusion of the nonionic detergent pluronic F127 was essential if Fura-2 fluorescence ratios were to be recorded. For some experiments the cultured epithelia were also loaded with BAPTA by including the AM form of this compound (50 μM) in the Fura-2-AM-containing medium.

Measurement of $[Ca^{2+}]_i$ and I_{SC}

Membranes bearing Fura-2-loaded epithelia were mounted in a miniature Ussing chamber attached to the stage of an inverted microscope (Nikon TE300). Initially, the epithelia were maintained under open circuit conditions while transepithelial potential difference (p.d.) was monitored. Once this had stabilized (20–30 min), p.d. was clamped to 0 mV and the current required to maintain this potential (short-circuit current, I_{SC}) continuously displayed and recorded directly to hard disk using a voltage-clamp amplifier (VCC 600; Physiologic Instruments, San Diego, CA). Transepithelial resistance was calculated by measuring the currents flowing in response to brief excursions (1 mV) from this holding potential. Transepithelial p.d. is expressed with respect to the basolateral solution. Positive currents are displayed as upward deflections of the traces, and were defined as those carried by anions moving from the basolateral to the apical solution. While I_{SC} was monitored, the Fura-2 fluorescence ratio was also recorded (PTI RatioMaster fluorescence system, Photon Technology International, NJ) from an area in the center of the epithelium, containing 30–40 cells, which was viewed using a 40× extra long working distance objective (Nikon CFI Plan Fluor ELWD, 0.6 numerical aperture). This signal was also recorded to disk.

Data Analysis and Experimental Design

All nucleotides were added to the physiological salt solution bathing the apical side of the epithelia at a final concentration of 100 μM. Ionomycin and thapsigargin were also added to the apical bath at final concentrations of 0.1 μM and 1 μM respectively. Experimentally induced changes in $[Ca^{2+}]_i$ and I_{SC} were quantified by measuring each parameter at the peak of a response and subtracting the equivalent values measured immediately prior to stimulation. Pooled data are presented as means ± SE and values of n refer to the number of experiments in each group. The significance of differences between mean values was tested using Student's t-test.

Results

When grown on Transwell Col membranes, E/92/3 cells consistently ($n = 75$) became integrated into coherent, epithelial layers [*see also* 13]. Under open-circuit conditions, transepithelial resistance was 234 ± 9 Ωcm^2 and the cultured epithelia generated a p.d. of −0.2 ± 0.04 mV. Basal I_{SC} was 0.01 ± 0.001 μAcm^{-2}.

Relationship Between $[Ca^{2+}]_i$ and I_{SC}

Apical ATP ($n = 6$) increased I_{SC} (24.5 ± 2.8 μAcm^{-2}) and $[Ca^{2+}]_i$ (0.21 ± 0.03 ratio units). The peak change in I_{SC} occurred 18.1 ± 0.9 sec after switching to the ATP-containing solution while the $[Ca^{2+}]_i$-signal reached its peak 2.3 ± 0.4 sec later. Moreover, although the rise in $[Ca^{2+}]_i$ was well maintained, I_{SC} rapidly fell towards its basal value despite the continued presence of ATP (Figs. 1 and 3). Essentially identical responses were seen in

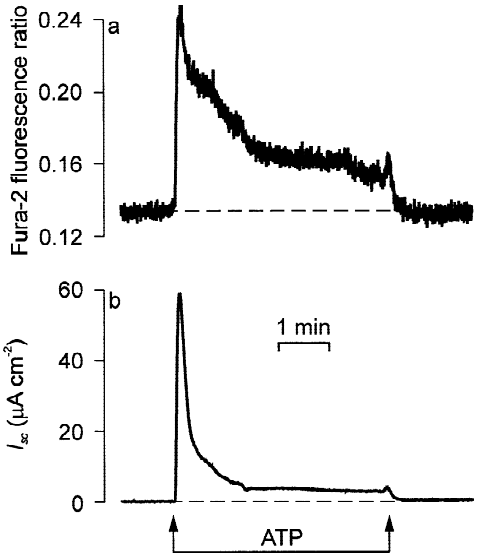

Fig. 1. The effects of apical ATP (100 μM) upon $[Ca^{2+}]_i$ (*a*) and I_{SC} (*b*) recorded from Fura-2-loaded epithelia. Essentially identical responses were obtained in 6 instances.

epithelia stimulated with UTP ($n = 4$), 5-Br-UTP ($n = 4$) or UDP ($n = 4$).

ATP also increased I_{SC} (42.8 ± 7.6 μAcm^{-2}) and $[Ca^{2+}]_i$ (0.29 ± 0.16 ratio units) in the absence of external Ca^{2+} (Fig. 2). The peak magnitude of these responses did not differ significantly from control but both signals fell rapidly to their respective basal values under these conditions (Fig. 2). Restoring apical Ca^{2+} elicited a relatively small but clearly sustained rise in $[Ca^{2+}]_i$ (0.04 ± 0.002 ratio units) accompanied by a minimal increase in I_{SC} (1.2 ± 0.3 μAcm^{-2}). Although basolateral Ca^{2+} evoked a more robust $[Ca^{2+}]_i$-signal (0.07 ± 0.01 ratio units), this was also accompanied by a small and poorly maintained electrometric response (rise in I_{SC}: 6.2 ± 0.3 μAcm^{-2}).

The data in Fig. 3*A* confirm that UTP increases both $[Ca^{2+}]_i$ and I_{SC} and that the rise in $[Ca^{2+}]_i$ is more sustained than the increase in I_{SC}. During the later part of this experiment, however, cells were exposed to ATP in the continued presence of UTP; this additional stimulus had essentially no effect upon $[Ca^{2+}]_i$ or I_{SC}. However, experiments in which these nucleotides were administered in the reverse sequence (Fig. 3*B*), showed that UTP increased both parameters in ATP-stimulated cells (I_{SC}: 8.9 ± 1.3 μAcm^{-2}, $[Ca^{2+}]_i$: 0.29 ± 0.02 ratio units).

Effect of BAPTA

ATP ($n = 11$) increased $[Ca^{2+}]_i$ in BAPTA-loaded monolayers but the peak response (0.15 ± 0.03 ratio units) was only ~70% ($P < 0.05$) of control (0.21 ± 0.03 ratio units) and the time taken to attain this maximum

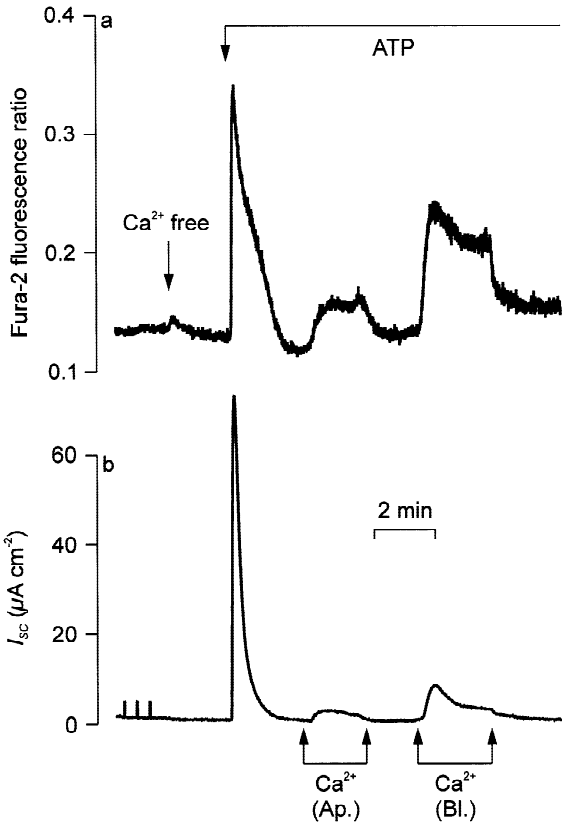

Fig. 2. Fura-2 loaded epithelia were initially superfused with control saline while $[Ca^{2+}]_i$ (*a*) and I_{SC} (*b*) were recorded. The cells were exposed to nominally Ca^{2+}-free solution as indicated and then stimulated with apical ATP (100 μM). Later in the experiment, the cells were exposed to pulses (~2 min) of external Ca^{2+} that were applied to the basolateral and then the apical aspects of the cultured epithelia ($n = 6$).

was prolonged ($P < 0.02$) to 156 ± 15 sec. Thereafter, however, $[Ca^{2+}]_i$ remained at this elevated level throughout the experiment. ATP also increases I_{SC} in BAPTA-loaded monolayers but the magnitude of this response (3.8 ± 1.4 μAcm^{-2}) was reduced to 20% of control. Although the rise in I_{SC} was also slowed (time to peak = 88 ± 6 sec) this effect was not as pronounced as the slowing of the $[Ca^{2+}]_i$ signal and so the peak change in I_{SC} occurred almost exactly a minute before the peak change in $[Ca^{2+}]_i$. BAPTA had qualitatively and quantitatively similar effects upon both components of the responses to UTP ($n = 10$), 5-Br-UTP ($n = 11$) and UDP ($n = 9$).

Effect of Thapsigargin

Thapsigargin elicited a slowly developing but sustained increase in $[Ca^{2+}]_i$ (0.23 ± 0.04 ratio units, $n = 11$) that was accompanied by a sustained rise in I_{SC} (7.51 ± 0.65 μAcm^{-2}). Subsequent application of ATP had no effect on $[Ca^{2+}]_i$ but caused a transient rise in I_{SC} (Fig. 5). Fur-

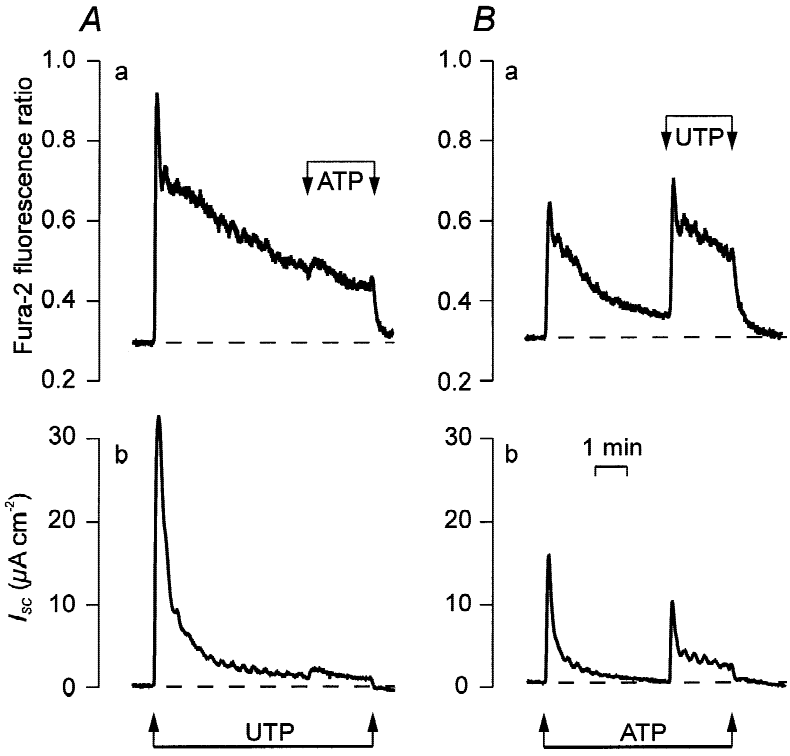

Fig. 3. Nucleotide-evoked changes in $[Ca^{2+}]_i$ (*a*) and I_{SC} (*b*) were recorded in cells stimulated with apical ATP and/or UTP (both 100 μM). (*A*) The effects of ATP in epithelia that had been previously exposed to UTP ($n = 5$). (*B*) The effects of UTP in epithelia that had previously been stimulated with ATP ($n = 6$).

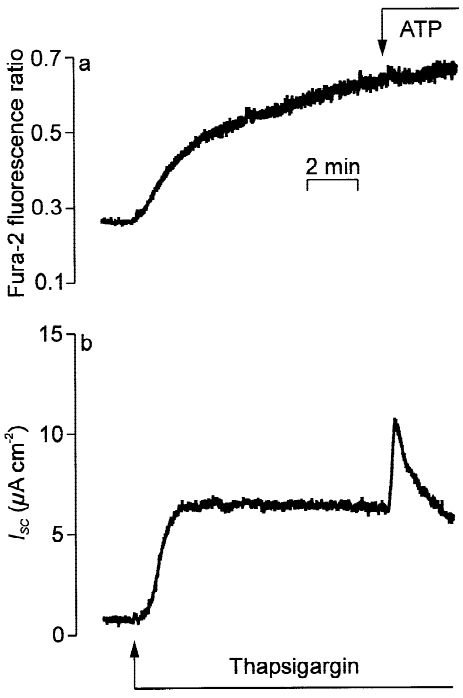

Fig. 4. Fura-2 fluorescence ratio (*a*) and I_{SC} (*b*) were recorded simultaneously from epithelia that were exposed to 1 μM thapsigargin (apical and basolateral) followed by 100 μM apical ATP ($n = 11$).

ther experiments showed that UDP (2.9 ± 1.2 μAcm^{-2}, $n = 6$) and UTP (3.3 ± 0.9 μAcm^{-2}, $n = 11$) also increased I_{SC} without affecting $[Ca^{2+}]_i$ in thapsigargin-treated cells. However, ionomycin increased both parameters under these conditions ($[Ca^{2+}]_i$: 0.04 ± 0.02 ratio units, I_{SC}: 8.5 ± 2.2 μAcm^{-2}, $n = 12$) confirming that changes in $[Ca^{2+}]_i$ could still be measured. In subsequent experiments, cells were exposed to thapsigargin under control conditions and external Ca^{2+} removed once the resultant increases in I_{SC} and $[Ca^{2+}]_i$ were established. Withdrawal of Ca^{2+} always caused a transient fall in I_{SC} (Fig. 5*A*). We can offer no explanation for this phenomenon but, once it had subsided, $[Ca^{2+}]_i$ decayed slowly towards its basal level. Subsequent application of apical ATP increased I_{SC} (4.8 ± 1.5 μAcm^{-2}) and this response was accompanied by slight acceleration in the rate at which $[Ca^{2+}]_i$ fell (Fig. 5*A*), suggesting that activation of the P2Y receptors in these cells enhances Ca^{2+} extrusion. This has been documented in other epithelia [37].

In a final series of experiments, epithelia were exposed to thapsigargin followed by ATP in the absence of external Ca^{2+}. Under these conditions thapsigargin elicited a transient rise in $[Ca^{2+}]_i$ (0.08 ± 0.02 ratio units) without a discernible rise in I_{SC} (Fig. 5*B*). Subsequent application of ATP elicited a slight fall in $[Ca^{2+}]_i$ that was consistently accompanied by a rise in I_{SC} (5.6 ± 1.0 μAcm^{-2}).

Discussion

The present data confirm that the apical P2Y receptors present in E/92/3 cells allow nucleotides to increase $[Ca^{2+}]_i$ [15] and I_{SC} [16, 35]. Our earlier studies suggested strongly that the electrometric responses were due

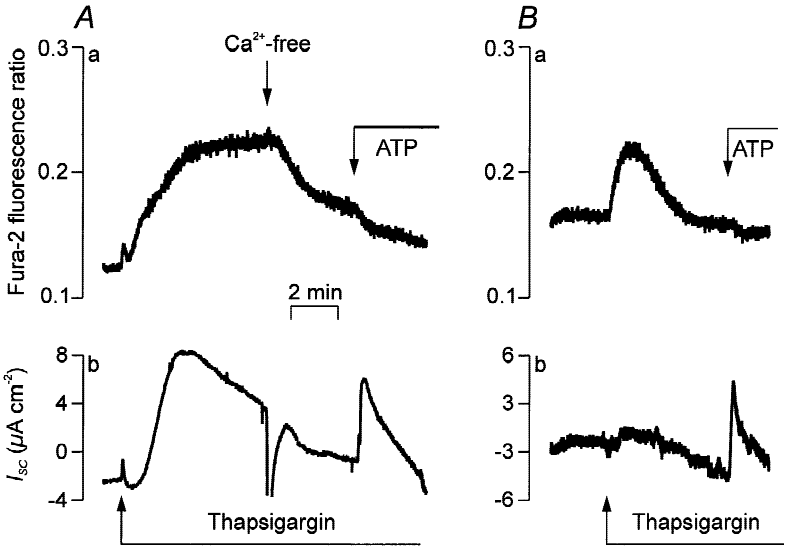

Fig. 5. Fura-2 fluorescence ratio (*a*) and I_{SC} (*b*) were recorded from cultured epithelia exposed to 1 μM thapsigargin and 100 μM apical ATP. (*A*) Cells were first exposed to thapsigargin under control conditions and external Ca^{2+} then withdrawn before the cells were exposed to ATP ($n = 5$). (*B*) Cells were continuously superfused with Ca^{2+}-free saline while being exposed to thapsigargin and ATP as indicated ($n = 4$).

to a nucleotide-evoked increase in apical anion conductance [16, 32], and so it was tempting to attribute the increased I_{SC} to $[Ca^{2+}]_i$-mediated activation of apical anion channels [*see* e.g., 3]. However, the new experimental approach used in the present study showed clearly that the $[Ca^{2+}]_i$ signals were more sustained than the changes in I_{SC}. It is, therefore, clear that the 2 components of the response have different time courses.

Experiments in which external Ca^{2+} was removed/replaced confirmed [15] that the nucleotide-evoked $[Ca^{2+}]_i$ signals were initiated by the mobilization of cytoplasmic Ca^{2+} but sustained by Ca^{2+} influx. Interestingly, although the receptors controlling Ca^{2+} entry were on the apical membrane, the Ca^{2+} influx occurred, primarily, across the basolateral membrane. Such assymetry has been reported in other epithelial cell types [6, 14] although not by all investigators [23]. However, the important point to emerge from these experiments was that Ca^{2+} entering nucleotide-stimulated epithelia had very little effect upon I_{SC}. This raised the possibility that the anion channels underlying the increases in I_{SC} may lose sensitivity to $[Ca^{2+}]_i$ during maintained stimulation. This has been seen in other cell types [20, 39] and would account for the transient nature of the present responses.

Cross desensitization experiments showed that, after I_{SC} had decayed back to its basal level, UTP could increase both $[Ca^{2+}]_i$ and I_{SC} in ATP-stimulated cells. This confirms [16, 33] that E/92/3 cells, in common with certain other epithelial cell types [10, 18, 19], express at least two $[Ca^{2+}]_i$-mobilizing P2Y receptor subtypes: $P2Y_2$ receptors that are equally sensitive to ATP and UTP [21] and 'pyrimidinoceptors' activated by UTP but essentially insensitive to ATP [5, 21]. Most importantly, however, these data show that the anion channels underlying the electrometric responses are not refractory to further stimulation, even after prolonged exposure to ATP. This result presented a paradox—if anion channels can be activated by a second, Ca^{2+}-mobilizing agonist, then why should I_{SC} decay to its basal value despite a sustained increase in $[Ca^{2+}]_i$? One possibility may be that the channels are sensitive to the rate at which $[Ca^{2+}]_i$ rises, rather than to the absolute level of $[Ca^{2+}]_i$, although we are unaware of any studies which report the existence of anion channels with this property. In an attempt to define the role of $[Ca^{2+}]_i$ more clearly, we loaded the cultured epithelia with BAPTA, a Ca^{2+} buffer, in an attempt to block nucleotide-evoked $[Ca^{2+}]_i$ signals. Although BAPTA inhibited and slowed the rise in $[Ca^{2+}]_i$, it did not totally abolish the response, which was surprising as this substance can eliminate nucleotide-evoked $[Ca^{2+}]_i$ signals in E/92/3 cells on coverslips [33]. BAPTA also slowed and inhibited the rise in I_{SC}, suggesting that changes in $[Ca^{2+}]_i$ play an important role in this response. However, the peak change in I_{SC} now occurred ~1 min before the peak of the $[Ca^{2+}]_i$ signal, suggesting strongly that $[Ca^{2+}]_i$ is not the only determinant of I_{SC}.

In further experiments we used thapsigargin, a substance that blocks Ca^{2+} signaling by inhibiting the Ca^{2+} pumping enzymes of the endoplasmic reticulum [*see* e.g., 29] to disrupt the mechanism permitting receptor-mediated control over $[Ca^{2+}]_i$ and, under control conditions, this substance caused sustained increases in $[Ca^{2+}]_i$ and I_{SC}. Thapsigargin also increased $[Ca^{2+}]_i$ in the absence of external Ca^{2+}, but this response developed slowly and was transient. Interestingly, thapsigargin had no effect upon I_{SC} in this situation, and so this response appears to be mediated entirely by Ca^{2+} entering the cell from the external fluid. This contrasts sharply with the nucleotide-evoked increases in I_{SC}, which seemed to predominantly dependent upon Ca^{2+} released from cytoplasmic stores. Discrepancies between the effects of thapsigargin and Ca^{2+}-mobilizing receptor agonists have been reported previously. Thapsigargin thus increases $[Ca^{2+}]_i$ in human sweat gland epithelial cells but has no effect upon transepithelial ion transport while Ca^{2+}-mobilizing

agonists, such as carbachol and ATP, increase both parameters [1, 24]. It is therefore clear that I_{SC} does not necessarily follow $[Ca^{2+}]_i$ in at least some epithelial cell types.

The studies of thapsigargin-treated cells also showed that nucleotides increased I_{SC} even after their capacity to raise $[Ca^{2+}]_i$ had been abolished. These substances thus control I_{SC} by activating both $[Ca^{2+}]_i$-dependent and $[Ca^{2+}]_i$-independent pathways, and this may explain why the changes in $[Ca^{2+}]_i$ and I_{SC} had different time courses. Although ATP also increases cellular cyclic AMP levels in these cells [15, 25], this second messenger does not modulate membrane permeability or increase I_{SC} [13, 34]. Moreover, UTP also evoked a $[Ca^{2+}]_i$-independent response and yet this nucleotide, in contrast to ATP, lowers cellular cyclic AMP levels [35]. It is therefore abundantly clear that cyclic AMP cannot mediate the $[Ca^{2+}]_i$-independent responses to nucleotides. Other workers have shown, however, that epithelial P2Y receptors allow nucleotides to control anion channel activity *via* a signaling pathway that does not require a diffusable second messenger. It has been suggested that this may reflect a G protein-mediated interaction between P2Y receptors and anion channels co-localized in the apical membrane [8, 9, 26–28]. It is now important to undertake experiments to establish the mechanism of this $[Ca^{2+}]_i$-independent pathway, as it may be an important component of a poorly understood mechanism that permits control of ion transport.

This study was made possible by awards from The British Council, The MRC-ROPA scheme, The Research Grants Council of Hong Kong and a Direct Grant for Research from The Chinese University of Hong Kong. The authors thank C.Y. Yip and C.Y. Luk for their skilled technical help and Drs. S.K. Inglis, H.L. McAlroy and A. Collett for their helpful comments and discussions.

References

1. Brayden, D.J., Pickles, R.J., Cuthbert, A.W. 1991. Ion transport in cultured epithelia from human sweat glands: comparison of normal and cystic fibrosis tissues. *Brit. J. Pharm.* **102:**57–64
2. Chan, H.C., Cheung, W.T., Leung, P.Y., Wu, L.J., Chew, S.B.C., Ko, W.H., Wong, P.Y.D. 1996. Purinergic regulation of anion secretion by cystic fibrosis pancreatic duct cells. *Am. J. Physiol.* **271:**C469–C477
3. Cliff, W.H., Frizzell, R.A. 1990. Separate Cl^- conductances activated by cAMP and Ca^{2+} in Cl^- secreting cells. *Proc. Natl. Acad. Sci. USA* **87:**4956–4960
4. Clunes, M.T., Collett, A., Baines, D.L., Bovell, D.L., Murphie, H., Inglis, S.K., McAlroy, H.L., Olver, R.E., Wilson, S.M. 1998. Culture substrate-specific expression of $P2Y_2$ receptors in distal lung epithelial cells isolated from foetal rats. *Brit. J. Pharm.* **124:**845–847
5. Communi, D., Boeymans, J.M. 1997. Receptors responsive to extracellular pyrimidine nucleotides. *Trend. Pharmacol. Sci.* **18:**83–86
6. Gordjani, N., Nitschke, R., Greger, R., Leipziger, J. 1997. Capacitative Ca^{2+} entry (CCE) induced by luminal and basolateral ATP in polarized MDCK cells is restricted to the basolateral membrane. *Cell Calcium* **22:**121–128
7. Grynkiewicz, G., Poenie, M., Tsien, R.Y. 1985. A new generation of Ca^{2+}-indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **265:**3440–3450
8. Guo, X., Merlin, D., Harvey, R.D., Laboise, C., Hopfer, U. 1997. Pharmacological evidence that calcium is not required for P2-receptor-stimulated Cl^- secretion in HT29-Cl.16E. *J. Membrane Biol.* **155:**239–246
9. Guo, X., Merlin, D., Harvey, R.D., Laboisse, C., Hopfer, U. 1995. Stimulation of Cl^- secretion by extracellular ATP does not depend upon increased cytosolic Ca^{2+} in HT-29.cl16E. *Am. J. Physiol.* **269:**C1457–C1463
10. Inoue, C.N., Woo, J.S., Schweibert, E.K., Morita, T., Hanaoka, K., Guggino, S.E., Guggino, W.B. 1997. Role of purinergic receptors in chloride secretion in Caco-2 cells. *Am. J. Physiol.* **272:**C1862–C1870
11. Kerstan, D., Gordjani, N., Nitscke, R., Greger, R., Leipziger, J. 1998. Luminal ATP induces K+ secretion via a $P2Y_2$ receptor in rat distal colonic mucosa. *Pfluegers Arch.* **436:**712–716
12. Knowles, M.R., Olivier, K., Noone, P., Boucher, R.C. 1996. Pharmacological modulation of salt and water transport in the airway epithelium in cystic fibrosis. *Am. J. Resp. Crit. Care Med.* **151:**S65–S69
13. Ko, W.H., Chan, H.C., Chew, S.B.C., Wong, P.Y.D. 1996. Ionic mechanisms of Ca^{2+}-dependent electrolyte transport across equine sweat gland epithelium. *J. Physiol. Lond.* **493:**885–894
14. Ko, W.H., Chan, H.C., Wong, P.Y.D. 1997. Anion secretion induced by capacitative Ca^{2+} entry through apical and basolateral membranes of cultured equine sweat gland epithelium. *J. Physiol. Lond.* **497:**19–29
15. Ko, W.H., O'Dowd, J.J.M., Pediani, J.D., Elder, H.Y., Bovell, D.L., Jenkinson, D.M., Wilson, S.M. 1994. Extracellular ATP can activate autonomic signal transduction pathways in cultured equine sweat gland epithelial cells. *J. Exp. Biol.* **190:**239–252
16. Ko, W.H., Wilson, S.M., Wong, P.Y.D. 1997. Purine and pyrimidine nucleotide receptors in the apical membranes of equine cultured epithelia. *Brit. J. Pharm.* **121:**150–156
17. Lazarowski, E.R., Homolya, L., Boucher, R.C., Harden, T.K. 1997. Direct demonstration of mechanically induced release of cellular UTP and its implication for uridine nucleotide receptor activation. *J. Biol. Chem.* **272:**24348–24354
18. Lazarowski, E.R., Paradiso, A.M., Watt, W.C., Harden, T.K., Boucher, R.C. 1997. UDP activates a mucosal-restricted receptor on human nasal epithelial cells that is distinct from the $P2Y_2$ receptor. *Proc. Natl. Acad. Sci. USA* **94:**2599–2603
19. McAlroy, H.L., Collett, A., Ahmed, S., Ko, W.H., Baines, D.L., Wilson, S.M. 1999. Multiple P2Y receptor subtypes in the apical membranes of Caco-2 colonic adenocarcinoma cells. *Proceedings of the Physiological Society* (*in press*)
20. Morris, A.P., Frizzell, R.A. 1993. Ca^{2+}-dependent Cl^- channels in undifferentiated human colonic cells (HT-29). II. Regulation and rundown. *Am. J. Physiol.* **264:**C977–C985
21. Nicholas, R.A., Watt, W.C., Lazarowski, E.R., Qing, L., Harden, T.K. 1996. Uridine nucleotide selectivity of three phospholipase C-activating P_2 receptors: identification of a UDP-selective, a UTP-selective and an ATP- and UTP-specific receptor. *Mol. Pharmacol.* **50:**224–229
22. O'Reilly, C.M., O'Farrell, A.M., Ryan, M.P. 1998. Purinoceptor activation of chloride transport in cystic fibrosis and CFTR-transfected pancreatic cell lines. *Brit. J. Pharm.* **124:**1597–1606
23. Paradiso, A.M., Mason, S.J., Zazarowski, E.R., Boucher, R.C.

1995. Membrane-restricted regulation of Ca^{2+} release and influx in polarized epithelia. *Nature* **377:**643–646

24. Pickles, R.J., Cuthbert, A.W. 1992. Failure of thapsigargin to alter ion transport in human sweat gland epithelia while intracellular Ca^{2+} concentration is raised. *J. Biol. Chem.* **267:**14818–14825
25. Rakhit, S., Murdoch, R., Wilson, S.M. 1998. Persistent desensitization of the β_2 adrenoceptors expressed by cultured equine sweat gland epithelial cells. *J. Exp. Biol.* **201:**259–256
26. Schwiebert, E.M., Egan, M.E., Hwang, T.H., Fulmer, S.B., Allen, S.S., Cutting, G.R., Guggino, W.B. 1995. CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. *Cell* **81:**1063–1073
27. Stutts, M.J., Chinet, T.C., Mason, S.J., Fullton, J.M., Clarke, L.L., Boucher, R.C. 1992. Regulation of Cl^- channels in normal and cystic fibrosis airway epithelial cells by extracellular ATP. *Proc. Natl. Acad. Sci. USA* **89:**621–1625
28. Stutts, M.J., Fitz, J.G., Paradiso, A.M., Boucher, R.C. 1994. Multiple modes of regulation of airway epithelial chloride secretion by extracellular ATP. *Am. J. Physiol.* **267:**C1442–C1451
29. Takemura, H., Hughes, A.R., Thastrup, O., Putney, J.W. 1989. Activation of calcium entry by the tumour promotor thapsigargin in parotid acinar cells. Evidence that an intracellular calcium pool, and not an inositol phosphate, regulates calcium fluxes at the plasma membrane. *J. Biol. Chem.* **264:**12266–12271
30. Taylor, A.L., Kudlow, B.A., Mars, K.L., Gruenert, D.C., Guggino, W.B., Schwiebert, E.K. 1998. Bioluminescence detection of ATP release mechanisms in epithelia. *Am. J. Physiol.* **275:**C1391–C1406
31. Ussing, H.H., Zerahn, K. 1951. Active transport of sodium as the source of electric current in the short circuited isolated from skin. *Acta Physiol. Scand.* **23:**110–127
32. Wilson, S.M., Ko, W.H., Pediani, J.D., Rakhit, S., Nichol, J.A., Bovell, D.L. 1995. Calcium-dependent regulation of membrane ion permeability in a cell line derived from the equine sweat gland epithelium. *Comp. Biochem. Physiol.* **111A:**215–221
33. Wilson, S.M., Law, V.W.Y., Pediani, J.D., Allen, E.A., Wilson, G., Khan, Z., Ko, W.H. 1998. Nucleotide-evoked calcium signals and anion secretion in equine cultured epithelia that express apical $P2Y_2$ receptors and pyrimidine nucleotide receptors. *Brit. J. Pharm.* **124:**832–838
34. Wilson, S.M., Pediani, J.D., Ko, W.H., Bovell, D.L., Kitson, S., Montgomery, I., Brown, U.M.O., Smith, G.L., Elder, H.Y., Jenkinson, D.M. 1993. Investigation of stimulus-secretion coupling in equine sweat gland epithelia using cell culture techniques. *J. Exp. Biol.* **183:**179–199
35. Wilson, S.M., Rakhit, S., Murdoch, R., Pediani, J.D., Elder, H.Y., Baines, D.L., Ko, W.H., Wong, P.Y.D. 1996. Activation of apical P_{2U} purine receptors permits inhibition of adrenaline-evoked cyclic AMP accumulation in cultured equine sweat gland epithelial cells. *J. Exp. Biol.* **199:**2153–2160
36. Wilson, S.M., Whiteford, M.L., Bovell, D.L., Pediani, J.D., Ko, W.H., Smith, G.L., Lee, C.M., Elder, H.Y. 1994. The regulation of membrane $^{125}I^-$ and $^{86}Rb^+$ permeability in a virally transformed cell line (NCL-SG3) derived from the human sweat gland epithelium. *Exp. Physiol.* **79:**445–459
37. Wolff, T., Leipziger, J., Fisher, K.-G., Klar, B., Nitschke, R., Greger, R. 1993. Evidence for agonist-induced export of extracellular Ca^{2+} in epithelial cells. *Pfluegers Arch.* **424:**423–430
38. Wong, P.Y.D. 1988. Control of anion and fluid secretion by apical P_2-purinoceptors in the rat epididymis. *Brit. J. Pharm.* **95:**1315–1321
39. Xie, W., Kaetzel, M.A., Bruzik, K.S., Dedman, J.R., Shears, S.B., Nelson, D.J. 1996. Inositol 3,4,5,6-tetrakisphosphate inhibits the calmodulin-dependent protein kinase II-activated chloride conductance in T84 epithelial cells. *J. Biol. Chem.* **271:**14092–14097
40. Zegarra-Moran, O., Sacco, O., Romano, L., Rossi, G.A., Galietta, L.J.V. 1997. Cl^- currents activated by extracellular nucleotides in human bronchial cells. *J. Membrane Biol.* **156:**297–305