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UTP-preferring P_2 receptor mediates inhibition of sodium transport in porcine thyroid epithelial cells

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1 The effects of adenosine 5′-triphosphate (ATP), uridine 5′-triphosphate (UTP) and analogues on forskolin-stimulated absorption of Na^+ by porcine thyroid epithelial cells were analysed in cultures grown as confluent monolayers on permeable supports in Transwell Ussing chambers.

2 85% of the forskolin (10 μM)-stimulated short-circuit current was inhibited by phenamil (1 μM), which is a selective antagonist for epithelial type Na^+ channels.

3 Phenamil-sensitive current was inhibited in a dose dependent manner by nucleotides added to the apical compartment of Ussing chambers. In contrast, the phenamil-resistant current, previously shown to represent anion secretion, was unaffected by nucleotides.

4 The order of potency (with EC_{50} values given in μM) was UTP (0.08) > > ATP (6.3) = uridine 5′-diphosphate (UDP) (6.6) > 2methyl-thio-adenosine-5′-triphosphate (2MeSATP) (84.5) > adenosine 5′-diphosphate (ADP) (147.8) > α,β-methylene ATP (> 150) > > adenosine (> 1000).

5 P_2 receptors mediating inhibition of sodium absorption were present on the apical membrane of the cells since addition of UTP (1 – 1000 μM) to the basal compartment of the Ussing chambers had little effect while subsequent addition to the apical compartment produced a normal response.

6 Cibachron blue (Reactive blue 2) (1 – 100 μM), an antagonist at some P_2 receptor subtypes, inhibited phenamil sensitive current in a dose dependent manner with half maximal inhibition occurring at 14.25 μM.

7 Suramin (100 μM), pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) (100 μM) and pyridoxal 5′-phosphate (P5P) (100 μM) showed only slight competitive antagonism against the response to UTP.

8 These results indicate that a UTP-preferring P_2 receptor located on the apical membrane of thyroid epithelial cells mediates inhibition of Na^+ absorption.

Keywords: Purinergic; P2U; epithelia; thyroid; sodium transport; UTP; Cibachron blue

Abbreviations: A23187, calcium ionophore A23187; ADP, adenosine 5′-diphosphate; α,βMeATP, α,β-methylene ATP; 2MeSATP, 2methyl-thio-adenosine-5′-triphosphate; ATP, adenosine 5′-triphosphate; ATPase, adenosine 5′-triphosphatase; CFTR, cystic fibrosis transmembrane conductance regulator; CIB, Cibachron blue = Reactive blue 2; cyclic AMP, adenosine 3′,5′-cyclic monophosphate; EC_{50}, concentration exerting a half-maximal effect; DMSO, dimethylsulphoxide; FRTL5 cells, transformed line of Fischer rat thyroid cells; G protein, guanyl nucleotide binding protein; HEPES, (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]); IP3, inositol 1,4,5-trisphosphate; I_{SC}, short-circuit current; P_2, class of purinergic receptors; P5P, pyridoxal 5′-phosphate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; TEP, transepithelial potential difference; TSH, thyroid stimulating hormone; UDP, uridine 5′-diphosphate; UTP, uridine 5′-triphosphate

Introduction

The thyroid gland epithelium possesses a bidirectional ion transport system with absorptive and secretory activities. Absorption is dependent on active transport of Na^+ (Bourke *et al*., 1987) while secretion is driven by secondary active transport of Cl^- (Armstrong *et al*., 1992). This system appears to be capable of controlling the volume of the follicles (Yap *et al*., 1991; 1993). We have documented the activation of parts of this system by adenosine 3′,5′-cyclic monophosphate (cyclic AMP) (Bourke *et al*., 1990; Armstrong *et al*., 1992) and inhibition by Ca^{2+} (Manley *et al*., 1988). The bidirectional transport system involves a variety of cation channels and a Cl^- channel on the apical membrane of the cells (Bourke *et al*., 1995; 1996), with an NaK2Cl symporter and Na^+/K^+ ATPase on the basolateral membrane (Armstrong *et al*., 1992).

There are many examples of hormones and diffusable mediators regulating Na^+ transport in epithelia, including a number of instances of purinergic regulation of epithelial ion transport (Harden *et al*., 1995; Burnstock 1995). Bennett *et al*. (1996) reported an effect of the combination of uridine 5′-triphosphate (UTP) and the sodium channel antagonist amiloride on mucociliary clearance from lungs in patients with cystic fibrosis, which was interpreted in terms of changes in secretory and absorptive dynamics in airway epithelium. The airways have a bidirectional ion transport system (Liedtke, 1989) with similarities to that of the thyroid epithelium (Armstrong *et al*., 1992).

In the thyroid, purinergic mechanisms are known to activate signalling cascades and modulate ionic conductances. Adenosine 5′-triphosphate (ATP) has been shown to activate phospholipase C in a transformed line of Fischer rat thyroid cells (FRTL-5) (Sato *et al*., 1992), and the Ca^{2+} phosphatidylinositol cascade in human (Raspe *et al*., 1991a) and dog thyroid (Raspe *et al*., 1991b). Efflux of Ca^{2+} was recorded in response to ATP in cultured human thyrocytes (Raspe *et al*., 1989). In FRTL-5 cells, ATP promoted efflux of iodide (Okajima *et al*., 1988) and Cl^- (Martin, 1992). In the intact follicles of guinea-pig thyroid slices *in vitro*, thyroid stimulating

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hormone (TSH) was found to enhance efflux of iodide (Manley *et al*., 1972).

However, regulation by purinergic mechanisms of the transepithelial active transport of salt by the thyroid epithelium appears not to have been described. In this paper we report that a UTP-preferring P_2 receptor inhibits Na^+ absorption by the thyroid epithelium, and speculate that this may play a role in co-ordination of opposing absorptive and secretory activities.

Methods

Culture preparation

Porcine thyroid cultures were established as previously described (Bourke *et al*., 1981; Armstrong *et al*., 1992). Briefly, about 30 porcine thyroid glands were collected at a local abattoir, cleared of fat and connective tissue, sliced, washed and subjected to enzymatic digestion for three 30-min periods at 37°C in Ca^{2+}- and Mg^{2+}-free Spinner's salt solution (Eagle, 1959; 100 ml per 100 g tissue) which contained 1 g neutral protease 1^{-1} with 0.1 g collagenase 1^{-1}.

Cells were separated by filtration through stainless steel mesh (0.5 mm) and centrifugation ($200 \times g$ for 10 min), and then washed three times by resuspension and centrifugation in incubation medium consisting of Minimal Essential Medium supplemented with (in mM): N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES) 20, L-glutamine 1, $NaHCO_3$ 10, nystatin (50,000 units 1^{-1}), gentamicin (50 mg 1^{-1}), porcine insulin (68 μM), 10% (v v^{-1}) heat-inactivated newborn calf serum and NaOH to adjust pH to 7.4.

The cells were cultured on Costar Transwells with collagen-coated transparent membrane supports 24.5 mm in diameter at 1×10^6 cells/well in 1.8 ml incubation medium at 37°C in a humidified atmosphere of 2.5% CO_2 in air. The medium was changed every 2 days. Cultures reached microscopically visible confluence within 4 days, and the transepithelial resistance increased to ~8,000 Ω• cm^2 over the next 3–7 days (studies reported were carried out between days 9 and 14).

Electrical measurements on Ussing chambers

The cells were changed into a simplified serum-free medium, composed of (in mM): NaCl 116, $NaHCO_3$ 10, KCl 4.4, KH_2PO 1, $CaCl_2$ 1.77, $MgS0_4.7H_20$ 0.81, glucose 5.55 and HEPES 20, requiring exactly 7.63 ml of 1 M NaOH/1 of medium to adjust the pH to 7.34 in the presence of CO_2 (2.5% in air). The Transwells were maintained under a humidified atmosphere of 2.5% CO_2 in air, at 37°C in a thermostatically controlled heated box.

Short-circuit current (I_{SC}), transepithelial potential difference (TEP), and resistance were recorded by a computer-controlled apparatus taking measurements every 10 s (Armstrong *et al*., 1992). Transwells were maintained with identical media in upper (4 ml) and lower (8 ml) chambers, at a controlled temperature of 37°C in a humidified atmosphere of 2.5% CO_2 in air. Electrodes were isolated from the Transwell by agar bridges (2% agar). Electrode chambers contained electrode buffer composed of (in mM): NaCI 150, KCl 5, and NaH_2PO_4 1, adjusted to pH 7.4 with NaOH. Agar bridges were equilibrated in electrode buffer. Transepithelial potential was recorded from Ag-AgCl half-cells connected to the upper and lower chambers by agar bridges 0.5 mm in diameter. Current was passed through platinum electrodes in 20-ml baths coupled to the upper and lower chambers by large (3-mm)-diameter agar bridges to isolate the culture from electrolytic products forming at the current electrodes.

Transwells were allowed to settle down for 15–20 min after installation in the apparatus and then forskolin (10 μM) was added to the basal compartment. After a spike of CI^- current lasting 7 min (Armstrong *et al*., 1992), the short-circuit current rose over 20–30 min to a plateau, predominantly of Na^+ current. After the current had stabilized, doses of test substances or control medium were added to develop cumulative dose-response curves. Nucleotides typically evoked

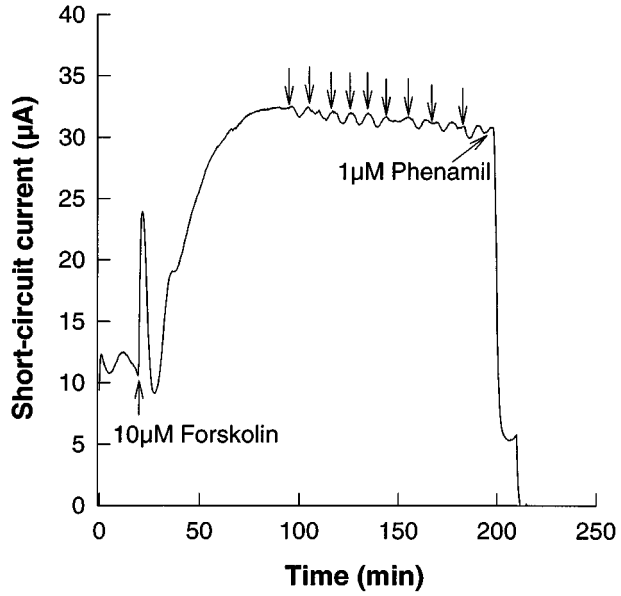

Figure 1 Response of the short-circuit current in a porcine thyroid epithelial cell monolayer cultured on a permeable support in a Transwell Ussing chamber to addition of forskolin (10 μM), multiple additions of control medium (arrows), followed by the sodium channel antagonist, phenamil (1 μM). Data shown are a representative experiment from six replications.

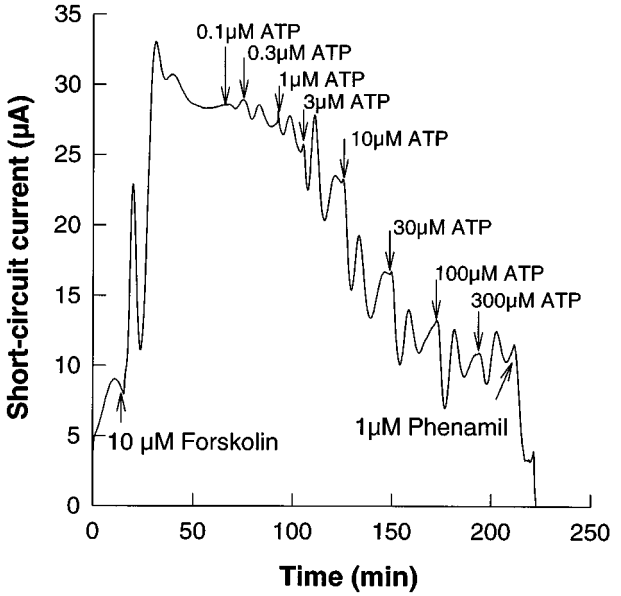

Figure 2 Response of the short-circuit current in a porcine thyroid epithelial cell monolayer cultured on a permeable support in a Transwell Ussing chamber to addition of forskolin (10 μM), followed after settling time by cumulative additions of adenosine triphosphate (ATP), and finally the sodium channel antagonist, phenamil (1 μM). Data shown are a representative experiment from seven replications.

a response with a damped train of oscillation: usually 12–15 min was needed for a steady state to be approached, and values recorded, before the next dose was added. At the completion of the series of test or control additions, phenamil (1 μM; a selective antagonist of epithelial type Na^+ channels) was added. Total Na^+ current was taken as the plateau values of short-circuit current minus the current remaining after phenamil. Na^+ current remaining in the presence of a test dose was taken as the total current in the presence of that dose minus the current remaining after phenamil. Data were expressed as the percentage inhibition of Na^+ current by test substance, and means $\pm$ s.e.mean of values from replicated experiments are reported.

The concentration of agonist producing a response which was 50% of maximal (EC_{50}) was determined by fitting dose-response data to a Michaelis-Menten type equation:

$$\text{Response} = \text{Maximal Response} * ([\text{agonist}]/EC_{50} + [\text{agonist}]).$$

The fitting was performed by the Marquard-Levenberg algorithm in the program SigmaPlot (version 1.02; Jandel Corporation, PO Box 7005, San Rafael, CA, U.S.A. 94912-7005). The coefficient of variation of the estimate of EC_{50} was always less than 2%, and the maximal response was in the range of 71–88% for purine and pyrimidine derivatives, except for α, β-methylene ATP and adenosine whose potency was too low for a full dose-response curve to be obtained. For these latter compounds, the data were interpreted by eye, and EC_{50} stated as greater than certain values.

Drugs and reagents

Neutral protease (dispase, grade II, 0.5 units mg^{-1}) was purchased from Boehringer Mannheim, Sydney, Australia, and collagenase (Worthington Type 1, 200 units mg^{-1}), Minimal Essential Medium, glutamine and nystatin from Flow Laboratories, Sydney, Australia. Newborn calf serum was purchased from ICN Biomedicals, Sydney, Australia. Costar Transwells with collagen-coated transparent membrane supports 24.5 mm in diameter (Transwell-COL 3425) were obtained from Costar, Cambridge, MA, U.S.A.

Phenamil, pyridoxalphosphate-6-azophenyl-2′, 4′-disulphonic acid (PPADS), pyridoxal 5′-phosphate (P5P) and suramin were purchased from Research Biochemicals International, (Natick, MA, U.S.A.). Phenamil was dissolved in dimethyl sulphoxide (DMSO; Sigma Australia, Castle Hill, NSW, Australia) as 10 mM stock solution. The maximal concentration of DMSO in the final incubation media was 0.1% vol vol^{-1}, appropriate control experiments showed this solvent to be without effect at this concentration. PPADS and P5P were dissolved in water. ATP, UTP and other mononucleotides were purchased from Sigma. All other reagents were analytical grade.

Results

Cultured porcine thyroid epithelial cells grown as a monolayer in Transwell Ussing chambers exhibited a basal positive TEP and I_{SC} which increased in a characteristic pattern on stimulation with cyclic AMP secretagogues, such as forskolin (Figure 1 and Armstrong *et al.*, 1992). After a biphasic rising zone, the plateau current remained constant or declined only slightly over 3 h (Figure 1). The majority of the current ($85 \pm 1.2\%$, mean $\pm$ S.E.M, $n = 13$) was inhibited by the antagonist phenamil, which has a high affinity and specificity for epithelial Na^+ channels (Figure 1).

Addition of purinergic agonists resulted in inhibition of the majority of the phenamil-sensitive I_{SC}. The responses had a

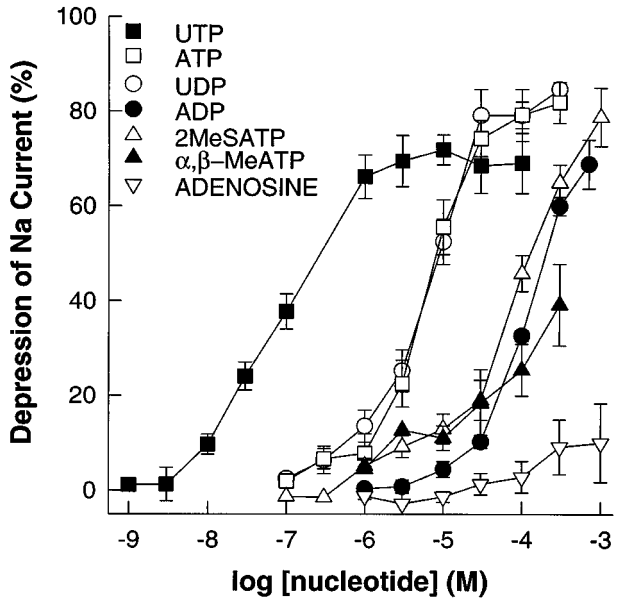

Figure 3 Inhibition of forskolin stimulated (10 μM) short-circuit current in porcine thyroid epithelial cell monolayers cultured on permeable supports in Transwell Ussing chambers by purines and pyrimidines. Data shown are cumulative dose response curves of the depression of short-circuit current expressed as a per cent of the total phenamil sensitive current (1 μM) (see Figure 2); means $\pm$ s.e.mean ($n = 5–10$). Compounds used were uridine 5′-triphosphate (UTP); uridine 5′-diphosphate (UDP); adenosine 5′-triphosphate (ATP); 2-methyl-thio-adenosine-5′-triphosphate (2MeSATP); adenosine 5′-diphosphate (ADP); α,β-methylene adenosine triphosphate (α,β-MeATP).

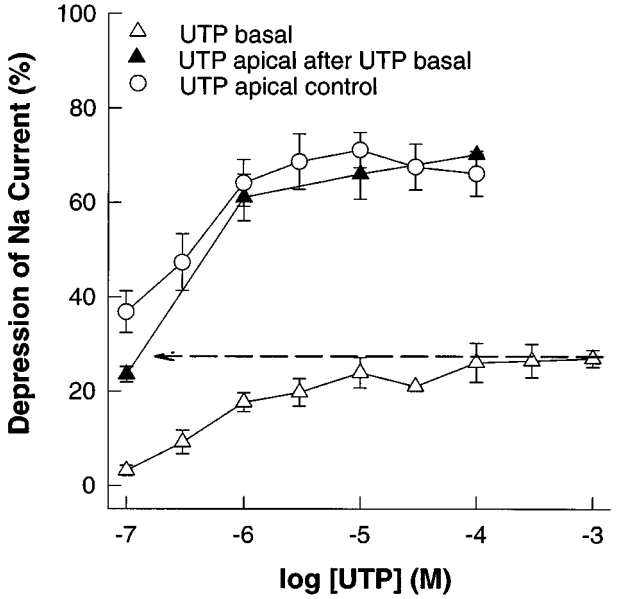

Figure 4 Inhibition of forskolin stimulated (10 μM) short-circuit current in porcine thyroid epithelial cell monolayers by uridine 5′-triphosphate (UTP) added to the basal, and then apical, compartments of Transwell Ussing chambers. Data shown are cumulative dose response curves of the depression of short-circuit current expressed as a per cent of the total phenamil sensitive current (1 μM) (see Figure 2). After completion of the cumulative dose response curve for UTP added to the basal compartment 1000 μM UTP remained in the basal compartment while doses of UTP commencing at 0.1 μM were added to the apical compartment. Data are means $\pm$ s.e.mean ($n = 6$).

characteristic damped oscillation, however it was practicable to obtain cumulative dose-response curves within 3 h (Figure 2 shows a typical response to ATP). Means of repeated experiments with purinergic agonists gave the following order of potency (with EC_{50} values given in μM) UTP (0.08) > > ATP (6.3) = uridine 5′ diphosphate (UDP) (6.6) > 2methyl-thio-adenosine-5′ triphosphate (2MeSATP) (84.5) > adenosine 5′ diphosphate (ADP) (147.8) > α, β-methylene ATP (> 150) > > adenosine (> 1000) (Figure 3).

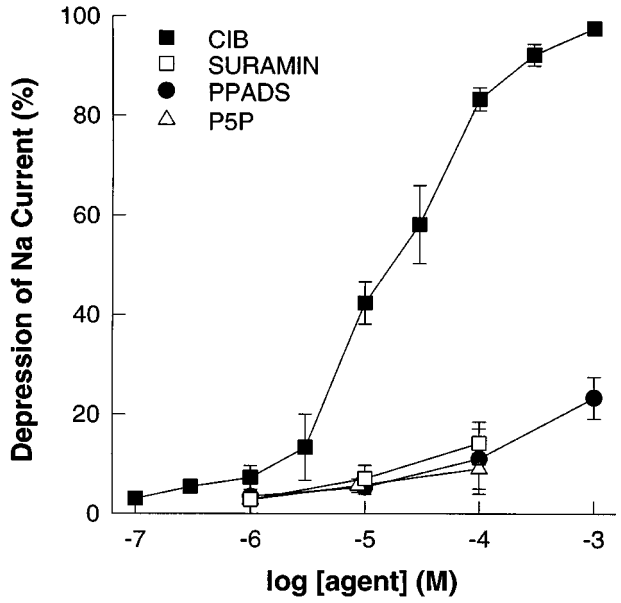

Figure 5 Effect on forskolin stimulated (10 μM) short-circuit current in porcine thyroid epithelial cell monolayers cultured on permeable supports in Transwell Ussing chambers of putative antagonists of purinergic receptors. Data shown are cumulative dose response curves of the depression of short-circuit current expressed as a per cent of the total phenamil sensitive current (1 μM) (see Figure 2). Compounds used were Suramin, Cibachron blue (Reactive Blue 2) (CIB), pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS); pyridoxal 5′-phosphate (P5P). Data are means ± s.e.mean (n = 4–6).

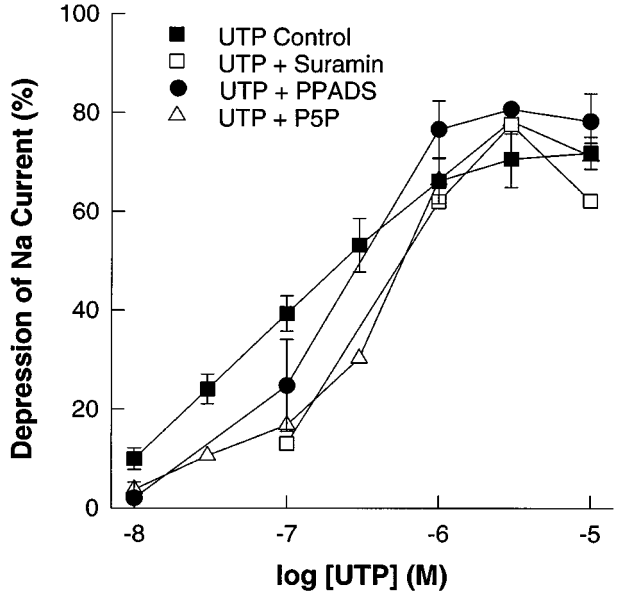

Figure 6 Effect of the purinergic receptor antagonists suramin (100 μM), pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) (100 μM) and pyridoxal 5′-phosphate (P5P) (100 μM) on the response to UTP of the forskolin stimulated (10 μM) short-circuit current in porcine thyroid epithelial cell monolayers cultured on permeable supports in Transwell Ussing chambers. Data shown are cumulative dose response curves of the depression of short-circuit current expressed as a per cent of the total phenamil sensitive current (1 μM) (see Figure 2). Data are means ± s.e.mean (n = 4–5 for UTP + antagonists, or n = 10 for UTP control).

Purinergic agonists were conspicuously more effective on addition to the apical than the basal compartment of the Transwells (Figure 4). Indeed, it was possible to add up to 1000 μM UTP to the basal compartment with only a modest effect, then obtain an essentially normal dose-response curve to UTP added to the apical compartment. It appears therefore, that the receptor mediating inhibition of Na^+ transport is located on the apical membrane of these polarized epithelial cells.

Cibachron blue (Reactive blue 2) which is an antagonist at a number of typical P_2 purinergic receptor subtypes (Burnstock, 1995), inhibited phenamil sensitive I_{SC} with an EC_{50} of 14.3 μM (Figure 5). Suramin, PPADS and P5P were essentially without effect alone (Figure 5) or as antagonists of the UTP response (Figure 6).

Discussion

The thyroid epithelium possesses opposing ion transport systems, which are able to alter follicular volume by secretion or absorption of ions and osmotically obliged water (Yap *et al*., 1991; Armstrong *et al*., 1992). Absorption of Na^+ through phenamil-sensitive epithelial Na^+ channels on the apical membrane of the cells (Bourke *et al*., 1996), followed by extrusion of Na^+ through the Na^+/K^+ ATPase on the basolateral membrane, drives fluid absorption, which reduces follicle volume (Yap *et al*., 1991). Conversely, when Na^+ absorption is blocked, secondary active transport of Cl^- drives fluid secretion (Armstrong *et al*., 1992), which increases follicle volume, and is essential for the formation of the lumen in reassembled thyroid follicles in culture (Yap *et al*., 1994). We have shown that altering follicle volume by osmotic challenge leads to a corrective response mediated by changes in ion transport (Yap *et al*., 1993), suggesting that this system plays a role in physiological regulation of follicle fluid dynamics.

Understanding of the regulation of such a complex system remains elusive, however. Activation of the cyclic AMP-dependent protein kinase A pathway results in immediate (seconds) activation of Cl^- secretion (Armstrong *et al*., 1992), through 6 pS Cl^- channels on the apical membrane (Bourke *et al*., 1995). Agents promoting cyclic AMP accumulation cause a slower (minutes) increase in short-circuit current due to Na^+ absorption (Armstrong *et al*., 1992). In studies of transepithelial movement of fluid as assessed by changes in the height of domes (detachments of the epithelial monolayer from the culture dish substrate; Bourke *et al*., 1987), we found that increasing intracellular Ca^{2+} with the Ca^{2+} ionophore A23187, inhibited Na^+ absorption (Manley *et al*., 1988). In addition to regulation of ion channels on the apical membrane, processes on the basolateral membrane may be important in activation of ion transport in response to cyclic AMP (Bourke *et al*., 1990) and follicle stretch induced by hypotonic media (Yap *et al*., 1993).

The present studies suggest a possible mechanism for co-ordination of opposing secretory and absorptive activities. It has been proposed that a mechanism involving (or linked to) the CFTR Cl^- channel releases ATP (al-Awqati, 1995; Cantiello *et al*., 1998). The Cl^- channels on the apical

membrane of thyroid epithelial cells have properties consistent with CFTR (Bourke *et al*., 1995). The P_2 receptor mechanism we describe here could mediate reciprocal regulation of Cl^- and Na^+ transport since the activation of secretion through Cl^- channels would lead to P_2 receptor-mediated inhibition of absorption through Na^+ channels. The P_2 receptor in these cells is on the apical membrane, where it will potentially be exposed to nucleotides released on activation of apical membrane Cl^- channels.

The classification of receptors responding to nucleotides is complex and evolving rapidly. Cloning of a number of receptors has placed on a firm footing the classification of purinergic receptors into G protein-coupled P_{2Y} and intrinsic ion channel P_{2X} families (Fredholm *et al*., 1997).

There is evidence for a class of P_{2Y} receptors, which, although not constituting a separate molecular family, behave functionally as pyrimidinergic receptors, preferring UTP to ATP (Communi & Boeynaems, 1997). Communi *et al*. (1995) cloned a G protein-coupled receptor with this property, exhibiting 51% sequence identity with the human P_2Y_2 receptor. A functional pyrimidinergic receptor (Communi *et al*., 1996), initially classified as P_2Y_4 was, like the system described in the present studies, insensitive to suramin, and preferred UTP. However, it differed in that PPADS strongly inhibited the UTP response.

Cibachron blue (Reactive blue 2) is an antagonist at a number of typical P_2 purinergic receptor subtypes (Burnstock, 1995). However, in the present studies it inhibited phenamil sensitive I_{SC}. This effect may have been non-specific, or, if mediated *via* the receptor, would have involved stimulation instead of inhibition.

Although the present studies provide no evidence about the signal transduction cascade activated by the P_2 receptor, it has been shown in a number of cell types that UTP mobilizes intracellular Ca^{2+} through a G protein coupled receptor mechanism activating phospholipase C to produce inositol triphosphate (IP3) (Communi & Boeynaems, 1997). In the present data, the response to nucleotides showed marked oscillations, a phenomenon which has been widely observed in Ca^{2+} mediated responses to purinergic agents (Morley *et al*., 1994) and has been subjected to theoretical analysis (Sneyd *et al*., 1995). Since we have shown that increased intracellular Ca^{2+} inhibits Na^+ absorption (Manley *et al*., 1988), such a mechanism would be the leading hypothesis for testing in future work on the response of thyroid epithelial cells to nucleotides.

In conclusion, these studies demonstrate a UTP-preferring P_2 receptor, located on the apical membrane of thyroid epithelial cells. Activation of this receptor leads to inhibition of Na^+ absorption. If release of ATP were coupled to cyclic AMP dependent activation of secretion, this purinergic receptor mechanism could play a role in co-ordination of the opposing absorptive and secretory activities of the thyroid epithelium.

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