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Regulation of Ion Content in Primary Cultures from Reabsorptive Ducts of Human Sweat Glands Studied by X-ray Microanalysis

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ABSTRACT. **X-ray microanalysis was used to investigate whether cAMP- and/or Ca^{2+}-activated regulation of chloride and potassium efflux is expressed in primary cultures of sweat gland duct cells. The effects of extracellular UTP and ATP on the duct cells, and the signalling system involved in the response to ATP was also studied. Primary cultures from duct cells of human sweat glands responded to 1 μM carbachol, 2 μM of the Ca^{2+} ionophore A23187, or 5 mM 8-bromo-cAMP stimulation for 5 min, resulting in a decrease in cellular Cl and K concentrations. 50 μM 5-nitro-2-(3-phenylpropyl-amino)-benzoic acid (NPPB), a Cl^- channel blocker, can inhibit the decrease in Cl concentration induced by cAMP. Extracellular (200 μM) ATP caused a decrease of Cl and K in cultured duct cells, while (200 μM and 2 mM) UTP was ineffective. Both the phosphoinositidase C inhibitor U73122 (10 μM) and the absence of extracellular Ca^{2+} abolished the ATP-induced decrease in Cl and K content. Alloxan (1.25 mM), an adenylate cyclase inhibitor, had an inhibitory effect on the response to ATP. The decrease in K, but not in Cl, content in the cells elicited by ATP was blocked by prior incubation with 100 ng/ml pertussis toxin, indicating the coupling of ATP to pertussis toxin-sensitive G-proteins. In conclusion, both Ca^{2+}- and cAMP-dependent Cl^- permeability is present in primary cultures from duct cells of human sweat gland. The response to ATP can be mediated both by Ca^{2+}- and by cAMP-dependent pathways, and is coupled to pertussis toxin-sensitive G-proteins.**

Sweating is primarily regulated by acetylcholine, which acts via muscarinic receptors that are functionally coupled to phosphoinositidase C. This enzyme evokes the hydrolysis of a membrane phospholipid, phosphatidylinositol-4,5-biphosphate (PIP_2) and the hydrophilic product of this reaction, inositol 1,4,5- triphosphate ($InsP_3$). $InsP_3$ enters the cytoplasm and releases Ca^{2+} from intracellular stores. The increase in intracellular free Ca^{2+} is sustained by calcium influx from the extracellular compartment (30). Sweat secretion can also be evoked by adrenergic agonists, but the response is generally smaller than that to responses of muscarinic agonists. α-Adrenoceptor agonists act by stimulating PIP_2 hydrolysis and so increasing intracellular free Ca^{2+}, whereas β-adrenergic agonists activate adenylate cyclase and hence their effects are thought to be mediated by cAMP (26).

In the duct of the human sweat gland, Cl^- is reabsorbed via the so-called cystic fibrosis transmembrane conductance regulator (CFTR), a channel that is regulated by cAMP-dependent phosphorylation and by intracellular ATP, and, possibly, Ca^{2+}. Previous reports have shown that primary duct cultures react in a similar manner to carbachol (muscarinic agonist) and isoprenaline (β-adrenergic agonist) stimulation (3, 4). On the other hand, other studies have demonstrated that cultured duct cells are insensitive to methacholine and isoproterenol (e.g., 14).

ATP can increase Cl^- permeability in isolated sweat glands, although the pharmacology of this response is not known (26, 30). Extracellular ATP and UTP can increase intracellular free Ca^{2+} by releasing calcium from intracellular stores and sustaining subsequent calcium influx (16, 30). The rise in intracellular free Ca^{2+} appears to be mediated via a type 2-purine (P2) receptor (7, 9). ATP can also raise the cellular cAMP content, whereas UTP is ineffective (16, 30). Stimulation of Cl^- transport by nucleotides may have clinical signifi cance and these substances have been suggested as therapeutic agents to improve Cl^- secretion in patients with

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Abbreviations: EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; NPPB, 5-nitro-2-(3-phenylpropyl-amino)-benzoic acid; DPC, diphenylamine-2-carboxylate; U73122, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidine-dione.

cystic fibrosis (15).

X-ray microanalysis can be used for the study of ion transport in cultured cells. The method has the advantage that changes in several different ions can be studied simultaneously, and that inhomogeneities in cell cultures can be easily revealed (27). In the present study, it was investigated whether cAMP- and/or Ca^{2+}-activated Cl^- permeability are expressed in primary cultures of sweat gland duct cells. The effects of extracellular UTP and ATP on Na, Cl and K content in the duct cells were also addressed. In subsequent experiments, the signalling system involved in the response to ATP was studied.

MATERIALS AND METHODS

Isolation and primary culture of the sweat gland duct

Normal human skin samples were obtained from patients (20–50 years old) undergoing mastectomy or abdominal surgery. The skin sample was stored in Hank's balanced salt solution at 4°C. Subcutaneous fat was removed and the skin was chopped with sharp scissors. This liberates intact sweat glands from the surrounding collagen fibers. The glands were collected by insect needles under a transmitted or reflected light microscope. The gland was then incubated in 1 mg/ml collagenase type II in a pre-culture medium containing William's E medium, penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), insulin (10 μg/ml), transferrin (10 μg/ml), hydrocortisone (5 ng/ml), epidermal growth factor (10 ng/ml), trace element mix 0.5% (v/v; Gibco BRL/Life Technologies, Paisley, Scotland, UK; Cat No 10404-010) and 20 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES) for 30 min at 37°C.

The reabsorptive duct and the secretory coil were separated, and the junction between these two parts was removed, together with remaining collagen and fat cells. After separation, the duct was allowed to recover in the pre-culture medium for 45 min. The ducts were cultured in 25 cm^2 tissue culture flasks (Costar, Cambridge, MA, USA) containing the culture medium (1% foetal calf serum added to the pre-culture medium). When a cellular outgrowth was seen, several ml of the culture medium were added.

Transmission electron microscopy

The cultured tissue was fixed with glutaraldehyde (2.5%) in 0.1 M cacodylate buffer (pH 7.35) for 30 min, and washed with 0.1 M cacodylate buffer. Tissue was postfixed in osmium tetroxide (1%) for 30 min, dehydrated in a graded ethanol series, and then infiltrated with absolute ethanol containing increasing concentrations of Agar 100 resin (Agar Scientific, Stansted, UK). The resin was polymerized for two days at 60°C. Sections were cut, contrasted with uranyl acetate and lead citrate, and examined in a Hitachi H-7100 transmission electron microscope (TEM) at 75 kV.

X-ray microanalysis

After 3–7 days, the cultured cells were incubated with 1 ml dispase (Boehringer, Mannheim, Germany) for 30–45 min. Sheets of cells detached from the floor of the culture flasks were allowed to recover in culture medium, and then seeded out on 75 mesh titanium grids (Agar Scientific, Stansted, UK). The grids had been covered with a Formvar (Merck, Darmstadt, Germany) film and coated with a thin carbon layer. The grids were sterilized under ultraviolet light before use. The cells were allowed to attach and spread for 1–5 days at 37°C in a humidified atmosphere of 5% CO_2/95% air in a culturing chamber.

The cultured duct cells were rinsed with Krebs-Ringer buffer (pH 7.4) to remove the culture medium, and then stimulated for 5 min at room temperature with the following agonists: 1 μM carbachol, 2 μM calcium ionophore A23187, or 5 mM 8-bromo-cAMP (cyclic adenosine monophosphate). The effect of a Cl^- channel blocker, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) (10) was tested. The cell-covered grids were incubated for 1 min in 50 μM NPPB followed by 5 min in 50 μM NPPB + 5 mM 8-bromo-cAMP. As controls, unstimulated cells were used.

The cultured cells were also stimulated with 200 μM UTP, 2 mM UTP, and 200 μM ATP for 5 min at room temperature. Unstimulated cells were used as controls. In later experiments, the cells were stimulated by 200 μM ATP under the following conditions: [1] pre-incubation (15 min) of 10 μM 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidine-dione (U73122), a phosphoinositidase C inhibitor (32). [2] in Ca^{2+}-depleted buffer supplemented with 5 mM EGTA. [3] in the presence of 1.25 mM alloxan, an adenylate cyclase inhibitor. [4] pre-treatment (24 h) with 100 ng/ml pertussis toxin, a G_o and G_i family G protein inhibitor (27). Untreated cells incubated under the same conditions were used as controls.

The incubation of the cell cultures grown on the Formvar-coated grids was stopped by rinsing the grids for 10 s in 0.15 M ammonium acetate to remove the salt-rick Krebs-Ringer solution. After the rinsing, the grids were blotted on filter paper and frozen in liquid propane cooled with liquid nitrogen. Frozen cell-covered grids were freeze-dried at −80°C in vacuum (10^{-5} torr) overnight. The freeze-dried cell-covered grids were coated with a conductive carbon layer before analysis.

X-ray microanalysis was performed at 100 kV in the transmission mode of a Philips (Eindhoven, The Netherlands) 400 transmission electron microscope with a LINK QX200 (Oxford Instruments, Oxford, UK) energy-dispersive X-ray microanalysis system. A few analyses were carried out on a Hitachi-7100 (Tokyo, Japan) electron microscope with an Oxford Instruments ISIS system. Quantitative analysis was carried out based on the peak-to-continuum ratio after correction for extraneous background (24), and by comparing the spectra from the cells with those from a standard, consisting of a gelatine/glycerol matrix containing mineral salts in

known concentrations (24). The static electron beam used in X-ray microanalysis had a diameter of 1.7–2.8 μm. Only one spectrum was acquired from each cell and the analytical volume comprised a large part of a single cell. All analyses were carried out in the central part of the grid and in the middle of grid squares, to minimize the extraneous background. The analyses were carried out over the thickest part of the cell (containing the nucleus) to optimize the signal. Differences in thickness between different cells are relatively small and corrected for by the use of the peak-to-continuum ratio, which corrects automatically for differences in thickness.

When data are presented as % of control, they were calculated as the ratio of the elemental concentrations in treated cells and the mean elemental concentration determined in control cells. Student's t-test was used to evaluate the statistical significance of differences between means. These differences were considered significant when $p < 0.05$.

Chemicals

Unless otherwise stated above, all reagents were purchased from Sigma (St. Louis, MO, USA) or from Gibco BRL/Life Technologies.

RESULTS

Transmission electron microscopy of a primary duct cell culture (Fig. 1a), after conventional fixation and resin embedding, showed that the cells grew in a monolayer with normal subcellular structures. The cultured cells presented typical epithelial characteristics: microvilli, desmosomes, and keratin filaments (Fig. 1b). Transmission electron microscopy of freeze-dried cultured cells used for X-ray microanalysis is shown in Fig. 2.

Stimulation of the cultured cells (5 min) with carbachol, A23187, and cAMP resulted in significant decreases in the cytoplasmic concentration of Cl and K and insignificant changes in Na content (Fig. 3). The loss of Cl from the cells was blocked when the cells were first exposed to the Cl^- blocker NPPB for 1 min and then, in the presence of NPPB, to cAMP for 5 min (Fig. 3). However, stimulation with cAMP in the presence of NPPB resulted in a significant further decrease in K, as well as a significant increase in Na concentration (Fig. 3). Examples of spectra of stimulated and unstimulated cells are shown in Figure 4.

Stimulation of the cultured cells with either 200 μM or 2 mM UTP for 5 min did not induce any significant changes in cellular Na, Cl, and K levels (Table I). Exposure of the cells to ATP for 5 min resulted in a significant decrease in Cl and K concentrations (Table I).

The cells pre-exposed to U73122 (15 min) and subsequently stimulated with ATP in the presence of U73122 (5 min) did not show any significant changes in Na, Cl, and K concentrations (Fig. 5). The ATP-induced decrease in Cl and K contents was abolished when the cells were stimulated with ATP in Ca^{2+}-depleted buffer supplemented with EGTA (Fig. 4). Alloxan had an inhibitory effect on the response to ATP when the cells were preincubated with alloxan (1 min) and stimulated with ATP in the presence of alloxan for 5 min (Fig. 4). When cells were preincubated with pertussis toxin (24 h) and then stimulated with ATP in the presence of pertussis toxin for 5 min, the ATP-induced decrease in cellular K content was blocked, but the decrease in Cl was not affected (Fig. 5).

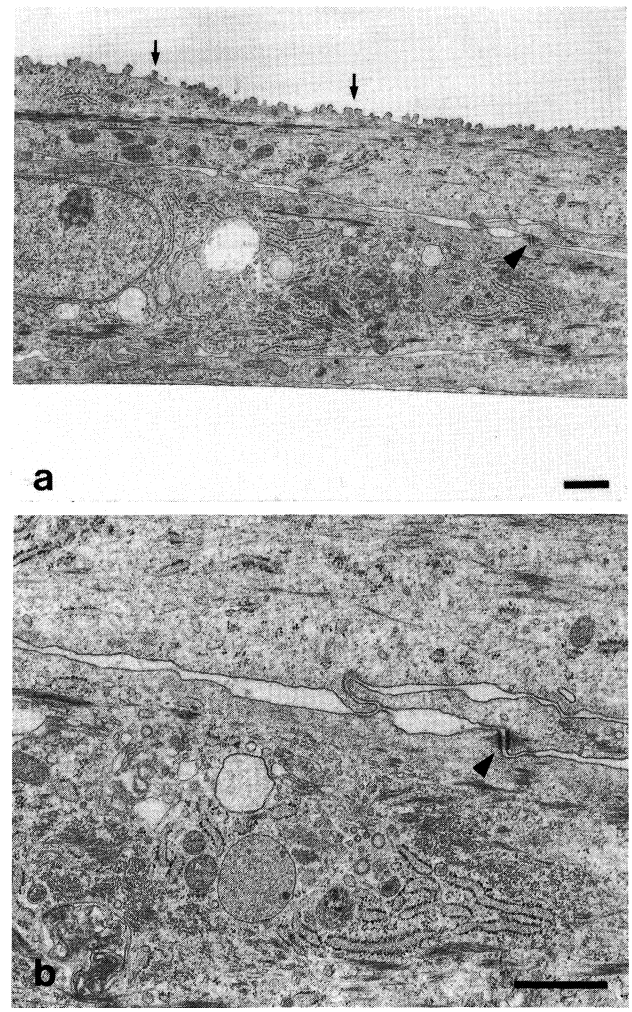

Fig. 1. Transmission electron micrograph of cultured human sweat gland duct cells (a) single layer cells grown on a plastic substrate, (b) enlargement of a part of (a) showing microvilli (arrows), desmosome (arrowhead). Scale bar, 1 μm.

DISCUSSION

Because of the obvious limitations in carrying out experiments on sweat glands in humans *in situ*, the use of

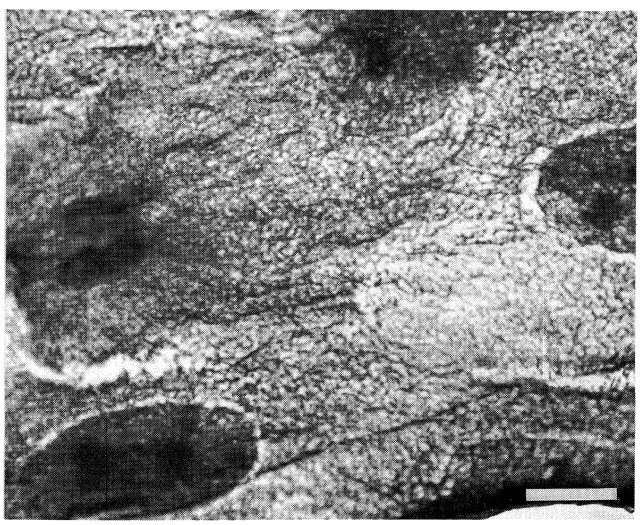

Fig. 2. Transmission electron micrograph of a freeze-dried specimen with cells from a primary culture of the human sweat gland duct grown on a Formvar film. Scale bar, 10 μm.

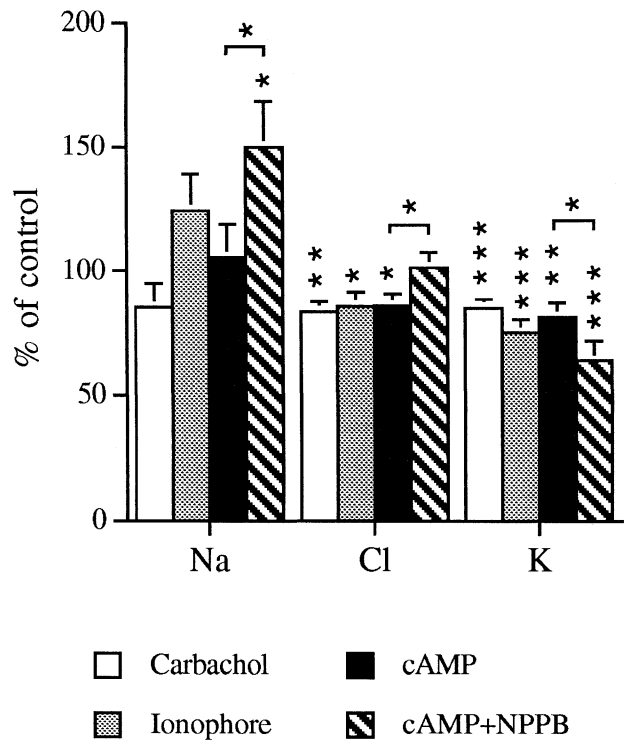

Fig. 3. Effect of carbachol, the calcium ionophore A23187, cAMP and the Cl^- channel blocker NPPB on cAMP stimulation. Data (in % of control) are given as mean and standard error (bars) of measurements. The cells were exposed to carbachol (5 min), A23187 (5 min), cAMP (5 min), or NPPB only (1 min) followed by UTP+NPPB (5 min). The statistical significance of the difference between trreated and control cells is indicated by asterisks. The brackets with asterisks indicate the significance of the difference between cAMP and cAMP+NPPB stimulation (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). The data are based on cultured cells from 5 human donors for the unstimulated control, and 3 donors for each of the treated experiments. From each donor about 10–15 cells were analyzed.

in vitro systems is indicated. We have recently shown that the ion content of sweat gland cells changes drastically during isolation of the gland, resulting in a smaller response to physiological activation, but the ionic content returns to normal in cultured cells (11). Also, it is difficult to obtain isolated sweat glands, in particular the duct part, for experiments. Therefore, it would seem better to use cell cultures rather than isolated glands for ion distribution experiments. Moreover, primary cell cultures have the advantage that the cells from the isolated glands can recover and multiply. Primary cultures of sweat gland duct cells show differences from *in situ* cells, but they clearly retain the structural characteristics of the epithelial cells, such as microvilli, keratin and desmosomes (14) as well as their physiological characteristics, such as cell polarity, i.e., the functional difference between apical and basolateral cell membranes (20).

Unlike the coil part of human sweat gland which is relatively easy to culture, the duct can be cultured with a success rate of only 20% (8, 11). This may be due to the fact that the duch appears to incur more damage than the coil during isolation, and many duct cells may not survive the isolation procedure. It is, however, possible that a few cells remain undamaged, and that these cells are propagated in primary culture (11). According to our present experience, the attachment of the explanted sweat gland on the substrate is one of the critical points for the success of the cell culture. In the present study, we have used a small amount of culture medium, and placed many isolated ducts together to allow the tissue to firmly attach onto the substrate. This increased the success rate. Moreover, because of the difficulty to obtain large numbers of tissue samples, all agonists were used at concentrations calculated to give maximal effects and no dose-response curves were obtained.

For X-ray microanalysis of cultured cells at the whole cell level, it is important to rinse the cells with a washing solution to eliminate the salt-rich incubation medium, which otherwise can disturb microanalysis. Rinsing the cells may induce dislocation of some diffusible elements (2, 12). However, the response to the washing solution is cell-specific. There is some difference of opinion regarding the best medium for rinsing; both distilled water and isotonic ammonium acetate which during freeze-drying evaporates without leaving a trace, are commonly used. In any case, published comparisons indicate no significant or only small differences between washing with ammonium acetate and distilled water (2, 11, 25, 29). Since unstimulated and stimu-

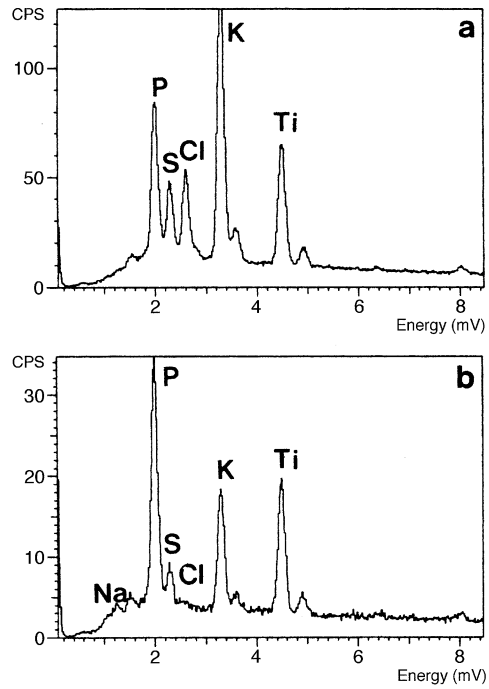

Fig. 4. Examples of energy -dispersive X-ray spectra of (a) unstimulated cells, and (b) cells stimulated with ATP. The peaks are denoted with their elemental symbols; the Ti peak is due to the grid.

Table I. EFFECT OF UTP AND ATP ON ELEMENTAL COMPOSITION IN PRIMARY CELL CULTURES OF HUMAN SWEAT GLAND.

	Unstimulated (n=63)	200 μM UTP (n=56)	2 mM UTP (n=25)	200 μM ATP (n=38)
Na	159±26	208±19	215±23	278±38
		P=0.16	P=0.20	P=0.14
Cl	184±9	195±8	212±6	156±12
		P=0.36	P=0.09	P=0.03
				*
K	555±25	502±25	585±45	425±40
		P=0.14	P=0.54	P=0.005
				***

Data given in mmol/kg dry weight, mean±standard error of mean; n = number of measured cells. The cells were exposed to UTP or ATP for 5 min at room temperature. The statistical significance of the difference between stimulated and unstimulated control cells is indicated by asterisks (* $P<0.05$; *** $P<0.001$). The data are based on cultured cells from 5 human donors for unstimulated controls, 4 donors with 200 μM UTP, 2 donors with 2 mM UTP and 3 donors with ATP. n = numbers of analyzed cells.

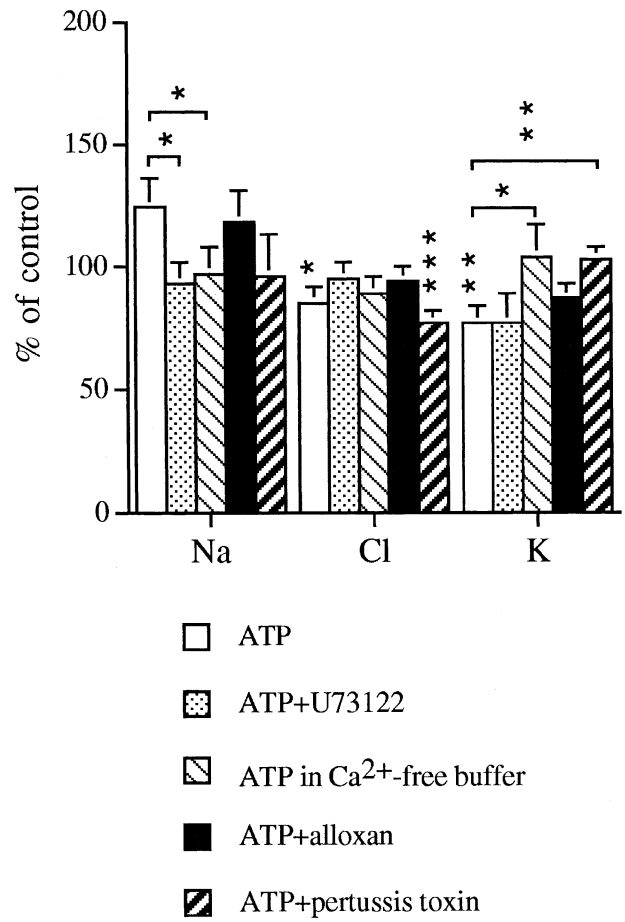

Fig. 5. Effect of the phosphoinosidase C inhibitor U73122, extracellular Ca^{2+}-free condition, the adenylate cyclase inhibitor alloxan, and pertussis toxin, an inhibitor for some subclasses of G proteins, on ATP stimulation. Data (in % of control) are given as mean and standard error (bars) of measurements. The cells were exposed to U73122 only (15 min) followed by ATP+U73122 (5 min), ATP in Ca^{2+}-free buffer supplemented with EGTA (5 min), alloxan only (1 min) followed by ATP+alloxan (5 min), or pertussis toxin only (24 h) followed by ATP+pertussis toxin (5 min). For controls, the cells were incubated under the same conditions, but without ATP. The statistical significance of the difference between treated and control cells is indicated by asterisks. The brackets with asterisks indicate the significance of the difference between stimulation with ATP in normal physiological buffer containing Ca^{2+} and that in Ca^{2+}-free buffer, or between stimulation with ATP only and that with ATP+pertussis toxin (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). The data for the response to ATP are derived from Table I. The other data are based on cultured cells from 3 human d9nors, except for U73122 with 2 donors. From each donor about 10–15 cells were analyzed.

lated cells are treated in the same way, it is unlikely that rinsing with ammonium acetate affects the final results in a noticeable way.

NaCl uptake by sweat gland duct cells involves passive diffusion of Na^+ across the apical membrane through an amiloride-sensitive pathway into the duct cell and an active extrusion of Na^+ from the cell across the basolateral membrane by the ouabain-sensitive Na^+-K^+-ATPase (1). K^+ ions can be secreted via basal K^+ channels. Cl^- ions move passively across both the apical and the basolateral membrane down their electrochemical gradient (22). This process is activated by

cAMP. In the present study the response of the cultured ducts to the stable cAMP analogue, 8-Br-cAMP, agrees with the findings of Pedersen (21), and suggests that this system has retained the characteristics of duch cells *in situ*. Moreover, a loss in Cl content evoked by cAMP in cultured duct cells was inhibited by the Cl^- channel blocker NPPB, suggesting an efflux of Cl^- ions via Cl^- channels. The inhibitory effect of NPPB on Cl^- permeability induced by appropriate agonists in cultured sweat gland has been previously shown (11, 18). In the present study, the effect of the Cl^- channel blocker NPPB (Fig. 3) seems more complicated. It appears that the combination of NPPB and 8-Br-cAMP may cause damage to the cell, which is consistent with the observed increase in Na and decrease in K. If NPPB would simply have had an inhibitory effect on Cl^- efflux, one would also have expected inhibition of the decrease in the cellular K content. It may be that this effect is "drowned" in the effect of the cell damage.

Our findings are in agreement with previous reports that primary duct cultures growing on permeable supports react in a similar way to carbachol and isoprenaline stimulation (3, 4). A similar situation has been observed in the human sweat gland cell line NCL-SG3, which secretes Cl^- in response both to cAMP and to an increase in intracellular Ca^{2+} (19, 23). On the other hand, when the cells were cultured on an impermeable substrate, primary cultures of duct cells were insensitive to methacholine and isoproterenol (14). It is plausible that the permeable substrate used in the present study allows full access of agonists to receptors on the basolateral surface of the cells (23). Another possibility is that the culture substrate affects the characteristics of the cultured cells. However, previous studies have shown that duch cells in isolated human eccrine sweat glands do not respond to muscarinic agonists (11, 13). This suggests that duct cells differentiate and may acquire coil cell characteristics during culture.

The present findings show that the Cl and K concentrations in cells from primary cultures from sweat gland ducts were sensitive to stimulation by ATP, but not by UTP. The effect of ATP on the Cl and K concentration has previously been shown in primary cultures from human sweat gland coils (18) and in a sweat gland cell line (30, 31). Our findings show that ATP, carbachol, the Ca^{2+} ionophore A23187, and cAMP had the same potency for activation of the loss of Cl from primary cultures of duct cells. A comparison of Figs. 3 and 5 shows that, on average, these four agonists caused a decrease of 20–25% of the Cl content of the cells. In primary cultures from human sweat gland coils, at 5 min after stimulation, the effect of ATP on the decrease of Cl concentration was approximately 50% of that of carbachol and about the same as that of acetylcholine (18). In NCL-SG3 cells, ATP is a very effective regulator of membrane permeability (30). The physiological basis of this discrepancy is unknown.

The effect of ATP was abolished by U73122 which inhibits phosphoinosidase C and in Ca^{2+} free medium, indicating that activation of ATP in primary cultures of duct cells is Ca^{2+}-dependent. Sato *et al.* (26) found that, in intact glands, the secretory response to ATP is less than 10% of that found with muscarinic agonists, but that in acutely dissociated cells, ATP and carbachol produce a similar increase in intracellular free Ca^{2+}. Alloxan, which can inhibit adenylate cyclase, had an inhibitory effect on the efflux of potassium ions stimulated by ATP in cultured duct cells. This suggests that ATP can activate an adenylate cyclase-cAMP-coupled mechanism. ATP had been shown to elicit cellular cAMP production in cultured cell lines from human (30) and equine (16) sweat glands.

Wilson *et al.* (30) demonstrated that extracellular application of ATP can increase the intracellular free Ca^{2+} concentration due to release first from an internal store, and subsequently by Ca^{2+} influx from the extracellular compartment. The effect appears to be mediated by the $P2_U$ receptor in the sweat gland cell line NCL-SG3. However, ATP may be catabolized by extracellular ATPases and nucleotidases on the cell surface to ADP and adenosine (9). It is possible that the response to ATP seen in the present study may be due, in part, to ADP (via purinergic receptors) and/or adenosine (via adenosine receptors). Moreover, at the high concentration of ATP (200 μM) used in this study, the possibility cannot be ruled out that adenosine (and adenosine metabolites), after uptake into the cultured cells, serve to dramatically replete intracellular purine nucleoside and nucleotide pools (reviewed in 5).

G_o and G_i are classes of G proteins that stimulate phosphoinositidase C and inhibit adenylate cyclase, respectively. The functional activity of both G_o and G_i can be inhibited by pertussis toxin (17). The present study shows that pertussis toxin inhibits the ATP-stimulated loss of K content, suggesting a mild effect of pertussis toxin on the response to ATP. Because ATP can activate adenylate cyclase, it is unlikely that ATP is coupled to G_i which inhibits this enzyme. G_o may therefore play a partial role in the response to ATP in primary cultures from duct cells. However, the possibility that the response to ATP in cultured duct cells may be mediated, in part, via pertussis toxin-insensitive G_q which also is coupled to phosphoinositidase C (17) cannot be ruled out.

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