Ion transport in cultured pig tracheal submucosal gland acinar cells studied by X-ray microanalysis

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Ion transport in cultured pig tracheal submucosal gland acinar cells studied by X-ray microanalysis. A-L. Zhang, G.M. Roomans. ©ERS Journals Ltd 1997.
ABSTRACT: The submucosal glands of the airway may contribute more to the airway fluid than the surface epithelium. The cellular mechanisms underlying the regulation of electrolyte and water transport in airway submucosal glands are, however, still poorly understood. Therefore, we attempted to establish a cell culture system to facilitate study of this regulation.

Acinar cells were isolated by enzymatic disaggregation from pig tracheal submucosal tissue and cultured on a plastic substrate coated with human placental collagen. The fine structure of the cells in confluent culture was studied by conventional transmission electron microscopy. The elemental content in resting cells and stimulated cells grown on a permeable substrate was studied by X-ray microanalysis.

The cultured cells retained structural characteristics (microvilli, secretory granules and desmosomes) of *in situ* epithelia. The total intracellular Cl and K concentrations significantly decreased after stimulation with the cholinergic agonist acetylcholine, the predominantly alpha-adrenergic agonist norepinephrine, or the beta-adrenergic agonist isoproterenol. Both ionomycin and 8-bromoadenosine 3':5'-cyclic monophosphate (8-bromo-cAMP) caused a marked decrease of the intracellular Cl, K and Na concentrations. Cl⁻ secretion induced by acetylcholine was inhibited by Cl⁻ channel blockers anthracene-9-carboxylic acid and 5-nitro-2-(3-phenylpropyl-amino)-benzoic acid (NPPB), but Cl⁻ efflux induced by 8-bromo-cAMP was blocked only by NPPB. The intracellular Cl and Na content significantly increased and the cellular K content markedly decreased after treatment with ouabain.

These results indicate that the cultured acinar cells maintained the structural and functional characteristics of *in situ* tissue and that this system is suitable for studying aspects of ion and water transport by the airway submucosal gland cells.
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The human airway is covered by a thin layer of secretion, which is critical for the normal function of the respiratory system. This airway secretion consists mainly of glycoproteins, electrolytes and water, and is produced by the surface epithelium and the tracheobronchial submucosal glands. There is evidence to indicate that the tracheobronchial submucosal glands contribute more to the airway secretion than the surface epithelium. Therefore, the role of the tracheobronchial glands in diseases such as cystic fibrosis (CF) may be more important than that of the surface epithelium. The evidence mentioned is the following: 1) in the respiratory system of healthy adult humans, the volume of the submucosal gland cells exceeds that of the surface mucous epithelial cells (goblet cells) by a ratio of about 40:1 [1]; 2) the only pathological change observed in postmortem examinations of newborns with CF is that the gland ducts are obstructed by viscous mucus; 3) in the normal human bronchus, the cystic fibrosis transmembrane regulator (CFTR), a Cl⁻ channel, which is defective in patients with CF, is expressed at very low levels in the surface epithelium, but at considerably higher levels in the submucosal glands [2]; and 4) rats have relatively few submucosal glands in the airway wall, and did not develop severe lung complications when a genetic animal model for CF was developed [3].

Although 95% of the airway secretion consists of water and electrolytes [4], little is known about the cellular mechanisms underlying fluid transport and their regulation in airway epithelium, and in particular, in the submucosal glands. The complex structure of the airway wall makes it difficult to interpret experiments carried out *in vivo*. Furthermore, the *in vivo* preparations do not allow investigators to study the cellular mechanisms of secretion by submucosal glands separately. Primary cultures of tracheobronchial submucosal glands have therefore been developed [5, 6]. Because the structure of pig respiratory tract closely resembles that of humans [7] and is easily accessible, in the present study we used isolated acinar cells from pig tracheal submucosal glands to initiate a cell culture.

It is widely accepted that the directional ion fluxes constitute the driving force for water transport in exocrine glands [8]. X-ray microanalysis was employed in

the present study to detect changes in elemental content in resting and activated cells. X-ray microanalysis has the advantage that the content of several different elements and changes therein can be studied simultaneously. This technique has been applied to cultured tumour cells, transformed cell lines, and primary cell cultures [9, 10].

The aim of this study was to investigate the possibility of setting up a culture of pig tracheal submucosal gland acinar cells to study ion transport at the cellular level by X-ray microanalysis.

Materials and methods

Materials and solutions

5-nitro-2-(3-phenylpropyl-amino)-benzoic acid (NPPB) and diphenylamine-2-carboxylate (DPC) were kind gifts from H.C. Englert, Hoechst AG. Unless otherwise stated, all drugs and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) or from Gibco BRL/Life Technologies (Paisley, UK). Krebs-N-2-hydroxy-ethylpiperazine-N'-2-ethanesulphonic acid (HEPES) solution (KHS, pH 7.4) consisted of 120 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 15 mM HEPES. Standard Ringer's solution (SRS, pH 7.4) contained 140 mM NaCl, 5 mM KCl, 5 mM HEPES, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 5 mM glucose.

Isolation of acinar cells

Pig tracheas were obtained from the local slaughterhouse and transported in ice-cold sterile KHS containing antibiotics (100 U·mL-1 penicillin, 100 µg·mL-1 streptomycin) to the laboratory. After two rinses in fresh sterile KHS containing antibiotics, the tracheas were cut longitudinally into three pieces. A piece of airway wall was mounted in a stretched position on a dissection tray, and bathed in Hank's balanced salt solution (HBSS, pH 7.4) containing 20 mM HEPES and antibiotics. After the surface epithelium was removed by sharp dissection, the submucosal tissue was dissected from cartilage and adventitia, collected in fresh HBSS, and minced with scissors. Tissue fragments were recovered by centrifugation (200 g, 10 min) and then incubated at 37°C in HBSS containing 20 mM HEPES, collagenase type IV (500 U·mL-1), pancreatic porcine elastase (6 U·mL-1), hyaluronidase (200 U·mL-1), deoxyribonuclease (10 U·mL-1), penicillin (100 U·mL-1), streptomycin (100 U·mL-1), gentamicin (100 µg·mL-1) and amphotericin B (2.5 µg·mL-1) [5–6]. After 2 h of disaggregation, the disaggregated tissue was recovered by centrifugation (200 g, 10 min), washed twice in cell culture medium containing 40% Ham's F12 medium, 40% Dulbecco's Modified Eagle Medium (DMEM), 20% foetal calf serum (FCS) and antibiotics in the concentrations described above.

Initiation of cell culture

The disaggregated tissue was resuspended in the same culture medium, acinar cell clumps were picked up under a dissecting phase contrast microscope, and 5–10 clumps were cultured in a human placental collagen-coated Petri dish at 37°C in 5% CO_2/95% air. After 24 h, the medium was replaced with serum-free medium composed of 49% Ham's F12 medium, 49% DMEM, 2% Ultraser G and antibiotics as described above. The medium was changed every 3 days. After 1 week, penicillin, streptomycin, and amphotericin B were omitted, and the concentration of gentamicin was reduced to 50 µg·mL-1.

Transmission electron microscopy (TEM)

Cells reaching confluence on plastic substrate and fragments (1 mm3) of tracheal submucosal tissue were fixed with glutaraldehyde (2.5%) in 0.1 M cacodylate buffer for 30 min, and washed in 0.1 M cacodylate buffer before postfixation in osmium tetroxide (1%) for 30 min. The cells were dehydrated in an ethanol series, and infiltrated in absolute ethanol containing increasing concentrations of Agar 100 Resin (Agar Scientific, Stansted, UK). The resin was polymerized overnight at 60°C. Sections were cut, contrasted with uranyl acetate and lead citrate, and examined in a Hitachi H-7100 transmission electron microscope (Tokyo, Japan).

Preparation of cultured cells for X-ray microanalysis

For X-ray microanalysis, after the culture reached confluence, cells were released from the substrate by 0.5% trypsin and then seeded onto 75 mesh titanium grids (Agar Scientific, Stansted, UK). The grids had been covered with a Formvar (Merck, Darmstadt, Germany) film coated with a thin carbon layer. The grids were sterilized under ultraviolet light before use. The cells were cultured on the grids for 4 or 5 days. In some experiments, after a quick rinse with SRS, the cells were incubated with SRS containing acetylcholine, norepinephrine, isoproterenol, ouabain, ionomycin or 8-bromo-adenosine 3':5'-cyclic monophosphate (8-bromo-cAMP) (table 1). In other experiments, the effect of different Cl- channel blockers: anthracene-9-carboxylic acid (9-AC) [11], NPPB [12], DPC [13] was tested. The cell-covered grids were preincubated with one of the Cl- channel blockers followed by incubation with acetylcholine or cAMP in the presence of this Cl- channel blocker (table 1). The dose of the agents used in this study is close to the known maximum dose for this or similar cell types [14]. As controls, we used cells incubated with only SRS (for 3 or 5 min), 1% dimethyl-sulphoxide (DMSO) in SRS, 1% ethanol in SRS, 0.5 mM 9-AC, 50 µM NPPB or 0.2 mM DPC (for 5 or 7 min). The incubation was terminated by a quick rinse with SRS and the cells were then washed with cold distilled water for 2–3 seconds to remove the salt-rich SRS. After the rinsing, the grids were quickly frozen in liquid propane cooled by liquid nitrogen (-180°C) and freeze-dried overnight in vacuum at -80°C. The freeze-dried specimens were covered with a conductive carbon layer before analysis.

Table 1. – Preincubation and incubation protocols of cultured pig tracheal submucosal gland cells

Preincubation			Incubation		
Added	Concentration μM	Time min	Added	Concentration μM	Time min
-	-	-	Acetylcholine	10	5
-	-	-	Norepinephrine	10	5
-	-	-	Isoproterenol	10	5
-	-	-	Ionomycin	3	3
-	-	-	8-bromo-cAMP	5	3
-	-	-	Ouabain	1	5
9-AC	500	2	Acetylcholine +9-AC	10 500	5
NPPB	50	2	Acetylcholine +NPPB	10 50	5
DPC	200	2	Acetylcholine +DPC	10 200	5
9-AC	500	2	8-bromo-cAMP +9-AC	200 500	3
NPPB	50	2	8-bromo-cAMP +NPPB	200 50	3
DPC	200	2	8-bromo cAMP +DPC	200 200	3

9-AC: anthracene-9-carboxylic acid; NPPB: 5-nitro-2-(3-phenylpropyl-amino)-benzoic acid; DPC: diphenylamine-2-carboxylate; 8-bromo-cAMP: 8-bromoadenosine 3':5'-cyclic monophosphate.

X-ray microanalysis

The specimens were analysed in a Hitachi H7100 electron microscope in the scanning-transmission electron microscopy (STEM) mode at 100 kV with an Oxford Instruments ISIS energy-dispersive spectrometer system (Oxford, UK). Quantitative analysis was carried out by determining the ratio of characteristic counts to the background under the peak (P/B), and by comparing P/B ratios from the cells with those from standards consist-ing of a gelatin/glycerol matrix containing mineral salts in known concentrations [15]. Spectra were acquired for 100 s. Only one spectrum was acquired from each cell.

Statistical analysis

All values are expressed as mean±SEM. Student's t-test was used to evaluate statistical significance. Significance was attributed to probability values of less than 0.05.

Results

Transmission electron microscopy of the cultured tracheal gland cells showed that the cells grew 1–3 cell layers thick. A representative image of the cultured cells is shown in figure 1. The epithelial characteristics of the cells were clearly demonstrated by the presence of microvilli and desmosomes. However, the cultured cells did not contain many secretory granules, in contrast to the acinar cells of the glands *in situ* (fig. 2). In the cells containing many mitochondria and an extensive Golgi complex, rough endoplasmic reticulum and free ribosomes were also present.

The average concentration of elements measurable by X-ray microanalysis in the cultured tracheal gland cells is given in table 2. The Ca content of the cultured cells was
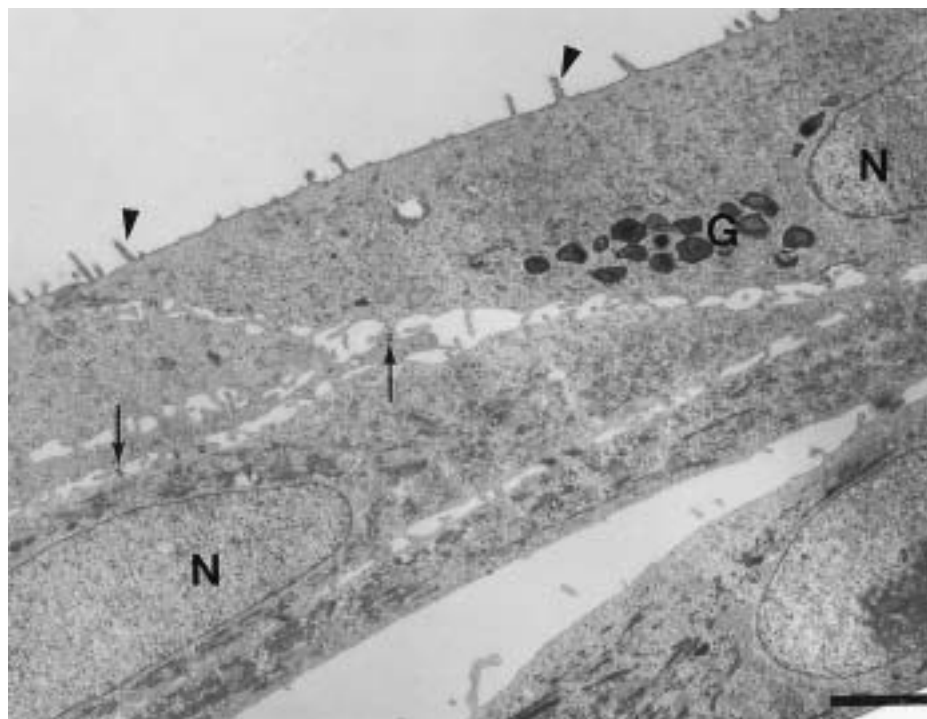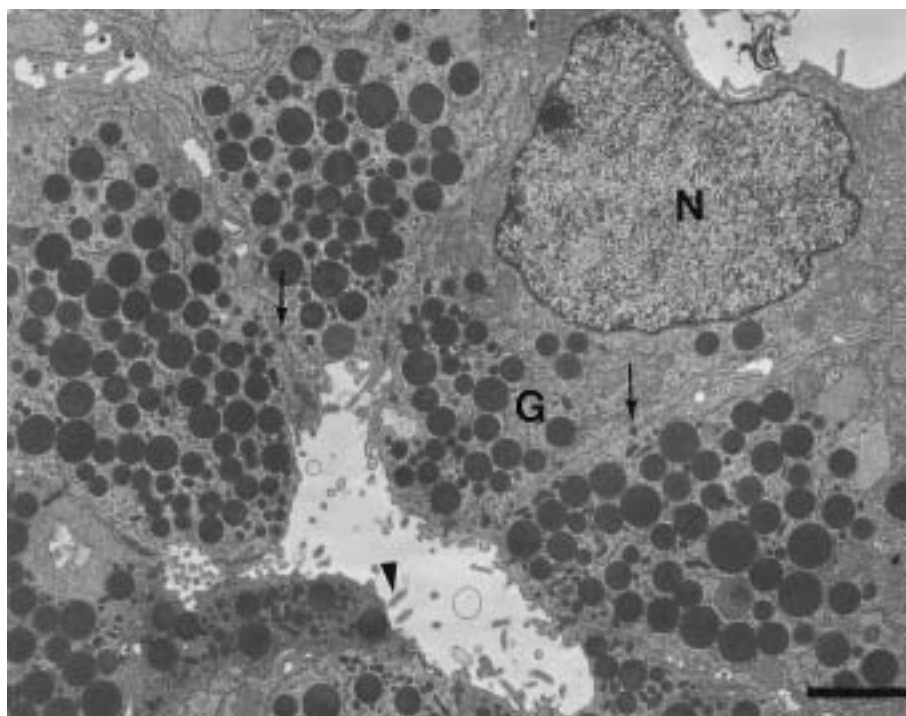

Fig. 1. – Transmission electron micrograph of cultured tracheal submucosal gland acinar cells. Desmosomes are indicated by arrows, microvilli by arrowheads. N: nucleus; G: secretory granules. (Internal scale bar=1.6 μm).

Fig. 2. – Transmission electron micrograph of *in situ* tracheal submucosal gland acinar cells. Desmosomes are indicated by arrows, microvilli by arrowheads. N: nucleus; g: secretory granules. (Internal scale bar=1.4 µm).

Table 2. – The elemental content of the cultured tracheal gland cells (control)

Element	Concentration mmol·kg dry weight^{-1}
Na	90±11
Mg	32±3
P	700±28
S	161±11
Cl	272±13
K	921±34

Results are given as mean±standard error. The data are based on four independent experiments using cell cultures set up from three pigs, in which 95 cells were analysed.
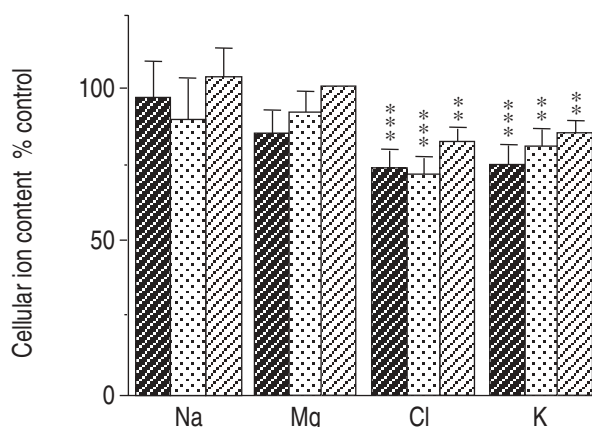

Fig. 3. – Effect of acetylcholine (▨), norepinephrine (⊡) and isoproterenol (▧) on the cellular content of Na, Mg, Cl and K in cultured pig tracheal submucosal gland acinar cells. The data are expressed as percentage of the control value. Thin bars indicate standard errors. The data are based on four experiments using cell cultures set up from 3 pigs. In each experiment, about 15 cells were analysed. **: p<0.01; ***: p<0.001, compared to control.

generally below the detection limit of the instrument.

The intracellular K and Cl concentrations significantly decreased after stimulation with acetylcholine, norepinephrine or isoproterenol, but no significant changes in the cellular content of Na and Mg were observed (fig. 3). Treatment with ionomycin or 8-bromo-cAMP caused a marked decrease of the cellular content of Na, Cl and K (fig. 4).

The intracellular Na and Cl content markedly increased while the cellular K content significantly decreased after 5 min of treatment with 1 mM ouabain (fig. 5).

A 30% decrease in the cellular content of Cl induced by acetylcholine was reduced to a 10% decrease by 9-AC, while the decrease in the intracellular K content was not affected. However, 9-AC did not block the Cl⁻ and K⁺ secretion induced by 8-bromo-cAMP (fig. 6). NPPB significantly inhibited the Cl⁻ and K⁺ secretion induced by acetylcholine or cAMP (fig. 6). DPC did not affect the changes in the cellular content of Cl and K elicited by either acetylcholine or 8-bromo-cAMP (not shown). The Cl⁻ channel blockers 9-AC, NPPB or DPC alone did not affect the elemental content of the cells (not shown). Also, DMSO or ethanol in the concentration needed to dissolve the Cl⁻ channel blockers and ionomycin did not cause significant changes in elemental content of the cells (not shown).
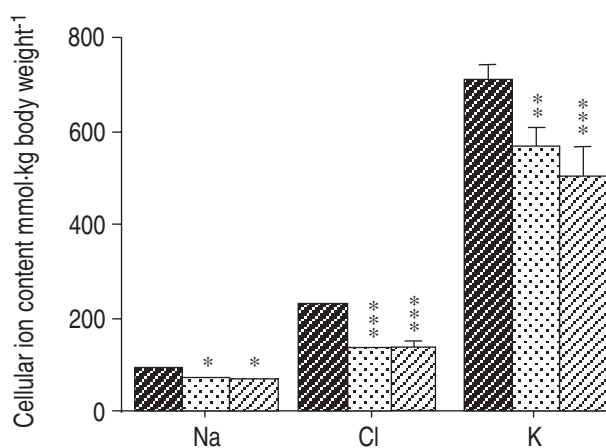

Fig. 4. – Effect of ionomycin and 8-bromo-cAMP on the cellular content of Na, Cl and K in cultured pig tracheal submucosal gland acinar cells. The data represent means of 59 measurements from three experiments for control cells and 30 measurements from two experiments (using cell cultures from two pigs) for treated cells. Thin bars indicate standard errors. ▨ : control; ⊡ : ionomycin; ▧ : 8-bromoadenosine 3':5'-cyclic monophosphate (8-bromo-cAMP). *: p<0.05; **: p<0.01; ***: p<0.001, compared to control.
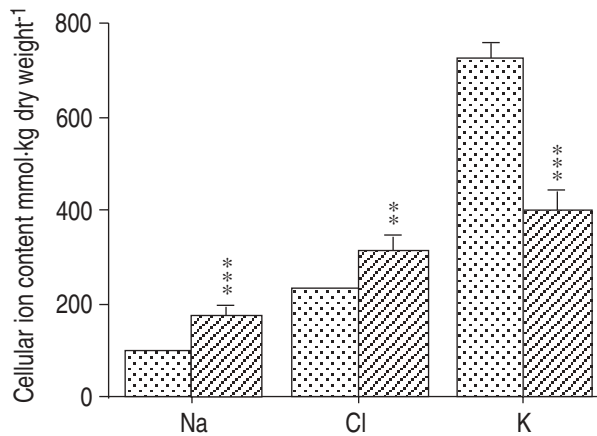
Fig. 5. – Effect of ouabain on the cellular content of Na, Cl and K in cultured pig tracheal submucosal gland acinar cells. Thin bars indicate standard errors. The data are based on three experiments (using cell cultures from two pigs). In each experiment, about 15 cells were analysed. ⬚ : control; ▨ : ouabain. **: p<0.01, ***: p<0.001, compared to control.
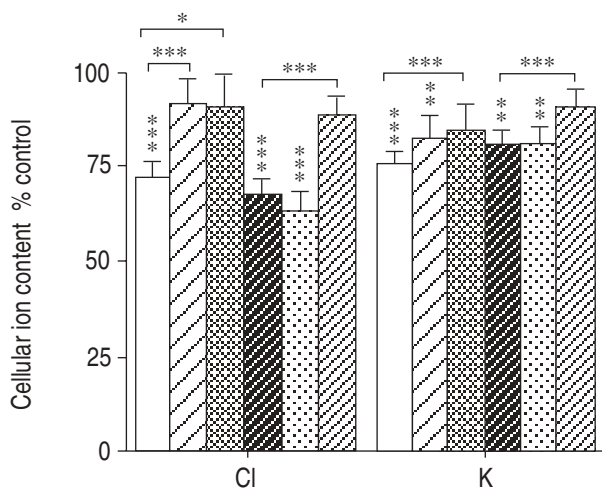

Fig. 6. – Effect of acetylcholine (ACh) and 8-bromo-cAMP, in the absence or presence of chloride channel blockers 9-AC and NPPB, on the cellular content of Cl and K in cultured pig tracheal submucosal gland acinar cells. The data are expressed as percentage of control value. Thin bars indicate standard errors. The data are based on two experiments (using cell cultures) from two pigs. In each experiment, about 15 cells were analysed. ☐ : ACh; ▨ : ACh+9-AC; ▨ : ACh+NPPB; ▨ : cAMP; ▨ : cAMP+9-AC; ▨ : cAMP+ NPPB. The brackets with asterisks indicate the significance of the difference between the stimulated cells and the stimulated cells in the presence of 9-AC or NPPB. Other asterisks indicate a statistical significant compared to control. *: p<0.05; **: p<0.01; ***: p<0.001.

Discussion

Fluid secretion from acini of major exocrine glands (e.g. pancreas and salivary glands) involves a series of ion movements. It is widely accepted that the active Cl⁻ secretion from the basal side of the epithelia to the acinar lumen and the accompanying passive Na⁺ flux build a local osmotic transepithelial gradient driving water into the lumen [8].

In this report, we demonstrate that the cholinergic agonist acetylcholine, norepinephrine (mainly acting on the alpha-adrenergic receptors) [16] and the beta-adrenergic agonist isoproterenol each induce a significant decrease of intracellular Cl⁻ concentration (fig. 3). The decrease induced by acetylcholine was almost completely inhibited by the Cl⁻ channel blockers 9-AC and NPPB (fig. 6). As with salivary glands [17, 18], cultured tracheal acinar cells released K⁺ after stimulation. K⁺ efflux across the basal membrane of acinar cells serves as a driving force by maintaining the cell membrane potential [8]. Our results are in agreement with one *in vivo* study [19] in which the secretory rate of the submucosal gland, after stimulation with each of three types of receptors agonists, was much greater than in unstimulated glands. UEKI *et al.* [20] presented similar effects of cholinergic and alpha-adrenergic receptor agonists, but did not report data on beta-adrenergic agonists. However, two *in vitro* studies using pieces of tracheal walls [21, 22] showed a different effect of beta-adrenergic agonist stimulation on fluid secretion. These studies reported that cholinergic and alpha-adrenergic agonists stimulated copious secretion whereas beta-adrenergic agonists had almost no effect. Another difference between results obtained in *in vivo* [18] and *in vitro* [21, 22] experiments is that a certain amount of baseline secretion was observed in the *in vivo* experiments, while almost none was found in the *in vitro* situation. Possibly denervation and loss of perfusion are responsible for these differences.

Based on Ussing chamber experiments, YAMAYA *et al.* [23] reported that cell cultures of human tracheal gland acini released Cl⁻ after stimulation with the cholinergic agonist methacholine, the alpha-adrenergic agonist phenylephrine or the beta-adrenergic agonist isoproterenol. It is presumed that cholinergic and alpha-adrenergic agonists act *via* a Ca²⁺-dependent pathway while beta-adrenergic agonists act *via* a cAMP-dependent pathway. To confirm the effect of second messengers on Cl⁻ secretion, we treated cell cultures with the calcium ionophore ionomycin and 8-bromo-cAMP, respectively, and found that both markedly reduced the cellular Cl and K concentrations (fig. 4). Although this is the first report directly demonstrating the existence of a cAMP-activated pathway for chloride secretion in the tracheal gland acinar cells [24, 25], this result is not surprising considering the relatively high level of expression of CFTR (a cAMP-dependent Cl⁻ channel) in human tracheobronchial submucosal glands [2, 26].

With freshly isolated acini or confluent cultures of enzymatically isolated acinar cells, two groups [27, 28] observed that cells responded to the cholinergic agonist carbachol by releasing Cl⁻ and that the cell response to carbachol was dependent on the ouabain-sensitive Na⁺-K⁺-ATPase pump in the basolateral membrane. In the present study, when the cells were exposed to 1 mM ouabain for 5 min, the expected effects of inhibition of the Na⁺-K⁺-ATPase were observed, namely loss of K and increase of the cellular Na and Cl concentrations (fig. 5).

Our results indicate that the cells in culture retained some of the morphological characteristics of *in situ* epithelia, and that the specific regulation of ion transport in the submucosal gland cell cultures still existed. We conclude that this system is suitable for studying aspects of ion and water transport by the submucosal gland cells, and that X-ray microanalysis is a convenient way to study simultaneous fluxes of several major ions.

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