J. Membrane Biol. 147, 295–304 (1995)

The Journal of
Membrane
Biology

Evidence for Na-K-Cl Cotransport in Alveolar Epithelial Cells: Effect of Phorbol Ester and Osmotic Stress

C. Clerici[1], S. Couette[1], A. Loiseau[1], P. Herman[2], C. Amiel[1]

[1]Department of Physiology and INSERM U 426, Faculté de Médecine Xavier Bichat, 16 rue Henri Huchard—BP 416 75870, Paris Cedex 18, France

[2]Laboratoire d'Otologie Expérimentale, Faculté de Médecine Lariboisière, 75010 Paris, France

Received: 14 February 1995/Revised: 15 June 1995

Abstract. We have investigated the presence of Na-K-Cl cotransport in alveolar type II cells using uptake of ^{86}Rb. Several data support the presence of a Na-K-Cl cotransport in these cells. First, a large fraction of ouabain-resistant ^{86}Rb uptake was inhibited by bumetanide and furosemide. Second, bumetanide-sensitive ^{86}Rb uptake required the presence of Na^+ and Cl^- in the incubation medium; dependency on extracellular Na^+ and K^+ was hyperbolic, with a Km of 14.6 mM and 8.3 mM, respectively, while dependency on extracellular Cl^- was sigmoidal, which suggests a 1:1:2 stoichiometry. Third, a fraction of amiloride-insensitive ^{22}Na influx was deeply inhibited by bumetanide. ^{22}Na influx was dependent on the presence of extracellular K^+ and Cl^-. Since Na-K-Cl activity dramatically decreased with time in culture, further characterization of the cotransport on polarized cells could not be performed. The phorbol ester PMA inhibited Na-K-Cl cotransport in a time- and concentration-dependent manner. This inhibition was mimicked by oleoylacetylglycerol, dioctanoylglycerol, and the diacylglycerol kinase inhibitor R59022, and was reversed by an antagonist of PKC, staurosporine. Since the Na-K-Cl cotransport has been reported to be involved in cell volume regulation, we investigated its modulation by changes in extracellular osmolarity. Na-K-Cl activity was increased after a two-step procedure: swelling in hypotonic medium followed by shrinking in hypertonic medium. Under these conditions, cotransport activity increased whenever PKC activity was up- or downregulated, which suggests that the cell volume-induced modulation of the cotransport is independent from the PKC activity. Though we were not able to determine the polarity of the cotransport, it may also be involved in the absorptive function of alveolar type II cells, and would provide an alternate pathway for sodium entry.

Key words: Sodium transport — Rubidium-86 influx — Sodium-22 influx — Cell volume — Protein kinase C

Introduction

The alveolar epithelium is an absorptive epithelia which actively transports sodium from the epithelial lumen to the plasma side. This sodium transport contributes to absorption of intraluminal fluid and maintains the alveolar air spaces relatively fluid-free (Basset, Crone & Saumon, 1987). There is considerable evidence indicating the presence of sodium transport mechanisms in alveolar type II epithelial cells. In tissue culture, the cells form domes which are dependent on the presence of sodium and which are inhibited by amiloride and ouabain (Goodman & Crandall, 1982). In addition, monolayers of alveolar type II cells actively transport sodium from the apical to the basolateral side under short circuit conditions. This active transport of sodium can also be inhibited by amiloride and ouabain (Mason et al., 1982; Cott et al., 1986).

A variety of membrane transport mechanisms may be involved in the transfer of sodium across the alveolar epithelium. Sodium ions entering the apical surface of the cell are extruded across the basolateral side by Na,K-ATPase. A number of processes are involved in the entry of sodium across the apical membrane. Amiloride-sensitive sodium channels have been identified in alveolar type II cells (Russo, Lubman & Crandall, 1992) and in apical membrane from alveolar type II cells (Matalon, Bridges & Benos, 1991). In addition, several sodium-

Correspondence to: C. Clerici

specific transports, usually present in other absorptive epithelia, have been characterized in alveolar type II cells: Na-H exchanger, Na-amino acid, Na-phosphate, Na-HCO_3 and Na-glucose cotransporters (Matalon, 1991). Besides, several other sodium transport mechanisms such as Na-K-Cl transport described in airway epithelia (O'Grady, Palfrey & Field, 1987) may also be present in alveolar epithelial cells.

The characteristics of the Na-K-Cl cotransport are very constant among the different epithelium. The Na-K-Cl cotransport is a cation-anion cotransport system with an absolute dependence upon the simultaneous presence of Na^+, K^+ and Cl^- (O'Grady et al., 1987). Another feature of Na-K-Cl cotransport is its sensitivity to "loop" diuretics, bumetanide and furosemide. In many cells Na-K-Cl cotransport activity is regulated by changes in cell volume (Strange, 1994) and by a number of cellular mediators including cyclic nucleotides, protein kinase C and specific receptor tyrosine kinase (O'Grady et al., 1987). While cell shrinkage leads to activation of the Na-K-Cl cotransport in most of the cells, the regulation of Na-K-Cl cotransport varies depending on cell and tissue.

The present study was undertaken to investigate the presence of Na-K-Cl cotransport in alveolar type II cells. We measured fluxes of ^{86}rubidium (Rb), a known tracer for K^+. The results demonstrate that a major fraction of ouabain-resistant ^{86}Rb influx occurs through a "loop" diuretic-sensitive Na-K-Cl transport. The present study also indicates that this Na-K-Cl transport activity is regulated by cell volume change induced by swell-shrink procedure and by intracellular mediators.

Materials and Methods

Materials

Reagents were obtained from the following sources: ^{86}RbCl (1–8 mCi/mg Rb), ^{22}Na from Amersham (Amersham, UK); phorbol 12-myristate 13 acetate (PMA), 1-oleoyl-2-acetyl-sn-glycerol (OAG), 1,2-dioctanoyl-sn-glycerol (diC8), 4α-phorbol 12,13-didecanoate (4α-PDD), isoproterenol, ouabain, ionomycin, furosemide, prostaglandin E_2, 8-bromoadenosine 3′, 5′-cyclic monophosphate (8-BrcAMP), 8-bromoguanosine 3′, 5′-cyclic monophosphate (8-BrcGMP), from Sigma (St Louis, MO); bumetanide from Hoffman-La Roche, the diacylglycerol kinase inhibitor R59022 was from Janssen Life Sciences Products (Olen, Belgium); Staurosporine and forskolin from Calbiochem (La Jolla, CA). Elastase from Worthington Biochemical (Freehold, NJ). Culture media and reagents were from Gibco-BRL (Paisley, United Kingdom). Plasticware was from Costar (Cambridge, MA).

Cell Culture

Alveolar type II cells were isolated from the lungs of adult Sprague-Dawley, specific-pathogen free rats, (200–250 g) as previously described (Clerici et al., 1992). Briefly, rats were anesthetized with 30 mg/kg i.p. of sodium pentobarbital and injected with 1 U/g i.v. of heparin sodium. After a tracheostomy was performed, the animals were exsanguinated and lungs were perfused via the pulmonary artery with solution II containing (mM): 140 NaCl, 5 KCl, 10 HEPES-Tris pH 7.5 (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffered with Tris (hydroxymethyl) aminomethane), 2 $CaCl_2$, 1.3 $MgSO_4$, 2.5 sodium phosphate buffer (Na_2HPO_4 and NaH_2PO_4, pH 7.5), pH 7.4 at 22°C. The lungs were then removed from the thorax and the airways were lavaged to total lung capacity (8–10 ml) 5 times with solution 1 containing (mM): 140 NaCl, 5 KCl, 10 HEPES-Tris, 2 EGTA (ethylene glycol-bis-(β-amino ethyl ether),N,N′-tetra-acetic acid), 2.5 sodium phosphate buffer, 6 glucose, pH 7.4 at 22°C to remove macrophages, and twice with solution II. Then lungs were filled with 12–15 ml of elastase solution (4 U/ml prepared in solution II) and incubated in a water bath in air at 37°C for 10 min, after which additional elastase solution was instillated for another 10 min incubation. The lungs were minced in a DNAase I solution (0.25 mg/ml), the enzymatic reactions were stopped with 5 ml of newborn calf serum. The tissular suspension was sequentially filtered through 150 and 30 μm nylon mesh. The filtrate was centrifuged at 130 × *g* for 8 min. The cell pellet was suspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mM D-glucose, and plated at a density of 10^6 cells/cm^2 in 25 cm^2 bacteriologic plastic dishes, at 37°C in a 5% CO_2 incubator for 1 hr to remove macrophages by differential adherence. The unattached cells were then removed and centrifugated at 130 × *g* for 8 min. The resulting cell pellet (70% purity and 90% viability) was plated at a density of 5×10^5 cell/cm^2 in either 24- or 6-well culture dishes. Culture medium was DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 10 μg/ml gentamicin, cells were incubated in a 5% CO_2/95% air atmosphere at 37°C. The cell purity, assessed using fluorescence with phosphine 3R, was 90 ± 2% after 22 hr incubation. Contaminating cells were principally macrophages.

Experiments were performed at 24 hr of culture. Culture medium was changed to serum-free medium consisting of DMEM containing 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin. Hormones or their antagonists, or their solvent were added at appropriate concentration in the culture medium and cells were incubated in their presence usually for 30 min unless otherwise specified. After that time, uptake studies were performed.

^{86}Rubidium Influx Studies

Measurements of rubidium (Rb) influx were performed according to a protocol modified from the method of Cheval et Doucet (1990). Assays were performed at 37°C in a *solution A* derived from Eagle's Minimal Essential Medium which contained (in mM): 120 NaCl, 5 RbCl, 1 $MgSO_4$, 0.15 Na_2HPO_4, 0.2 NaH_2PO_4, 4 $NaHCO_3$, 1 $CaCl_2$, 5 glucose, 4 nonessential amino acids, 20 HEPES. Osmolality was adjusted to 330 mosm/kg H_2O by mannitol addition and pH to 7.4. After removal of the culture medium, the cells were rinsed twice with 1 ml/well of the uptake solution and then were incubated with 0.5 ml *solution A* supplemented with 1 μCi/ml ^{86}RbCl in the presence or absence of inhibitors. Uptake was stopped by three washes with 1 ml/well of ice-cold rinsing solution containing (mM): 140 choline chloride, 3 $BaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 0.1% BSA, mannitol up to the same osmolality as the uptake solution, pH 7.4. Cells were air dried and were solubilized in 0.5% Triton X-100. Tracer activities were determined by liquid scintillation counting. Ouabain-sensitive ^{86}Rb influx (Os), calculated as the difference of ^{86}Rb influx in the absence and presence of 1 mM ouabain was used as an indicator of Na,K-ATPase

activity (Cheval & Doucet, 1990). The component of ouabain-insensitive ^{86}Rb influx which was inhibited by 0.25 mM bumetanide was used to estimate the activity of the Na-K-Cl cotransport system. The protein concentration was determined as described by Bradford (1976).

^{22}Na Influx Studies

Influx of ^{22}Na was assayed after depleting the cells of Na^+ by a 20-min incubation in a Na^+-free solution, NaCl being substituted by N-methyl-glucamine (NMG) for 20 min at 37°C. The solution was then changed to one consisting of (in mM): 14 NaCl, 35 KCl, 96 NMG, and 1 mM ouabain, supplemented with 2 μCi/ml ^{22}Na. Uptake was stopped and cells were processed as described above. When necessary, Na^+ and K^+ were substituted by NMG, and chloride by gluconate.

Effects of Polypeptide Hormones

The cells were preincubated for 30 min with 0.5 ml culture medium containing the compounds to be tested. Uptake was measured by removing the medium and adding 0.5 ml solution A containing 1 μCi/ml ^{86}Rb plus the hormone to be tested. At the end of incubation, cells were washed and uptake was performed in the presence of test agents.

Manipulations of Extracellular Osmolality

Osmolality of the extracellular solution was varied according to Rotin & Grinstein (1989). The cells were incubated following two procedures. The first consisted of a 30-min incubation in either solution A (330 mOsm/kgH_2O) or hypertonic medium (430 mOsm/kgH_2O), similar to *solution A* but with addition of sucrose prior to Or-Bs ^{86}Rb influx measurement. The second was a two-step 30 min procedure: the cells were exposed for 15 min to a hypotonic solution (230 mOsm/kgH_2O) which was identical to *solution A* except that NaCl concentration was reduced to 70 mM in order to adjust the osmolality to 230 mOsm/kg H_2O, following by a 15-min exposure with either *solution A* (isotonic) or a hypertonic solution composed of *solution A* containing mannitol or Na gluconate (430 mOsm/kg H_2O). Then Or-Bs ^{86}Rb influx was measured.

Presentation of the Data

Uptake of ^{86}Rb was expressed as nanomoles/mg protein. Results were presented as the mean ± SE of three to seven different experiments in which duplicates were obtained. One-way or two-way analyses of variance were performed by Fisher's least significant different test. Analysis of the kinetic data was performed by nonlinear regression model (logistic function).

Results

Time Course of ^{86}Rb Influx and Inhibition by Loop Diuretics

Initial experiments were performed to examine whether a loop diuretic-sensitive ^{86}Rb influx pathway was present in alveolar type II cells. Influx of ^{86}Rb was linear for at least 10 min (Fig. 1). In the presence of 1 mM ouabain ^{86}Rb influx was decreased from 213 ± 11 to 108 ± 9 nmoles/mg protein/5 min (Table 1). Bumetanide (0.25 mM) further decreased ^{86}Rb influx to 34 ± 4 nmoles/mg protein/5 min. These results indicate that in alveolar type II cells 49% of total ^{86}Rb influx is mediated by Na,K-ATPase and 35% through a bumetanide-sensitive pathway. The latter component represents 68% of the ouabain-insensitive ^{86}Rb influx (Table 1). Inhibition by bumetanide was concentration-dependent (Fig. 2). Half-maximal inhibition was achieved at 0.60 ± 0.27 μM. Another loop diuretic, furosemide, also caused a concentration-dependent inhibition of ouabain-insensitive ^{86}Rb influx with a 50% inhibitory concentration of 3.70 ± 0.38 μM (Fig. 2). The ouabain-resistant diuretic-insensitive component, which is presumed to reflect a leak pathway,

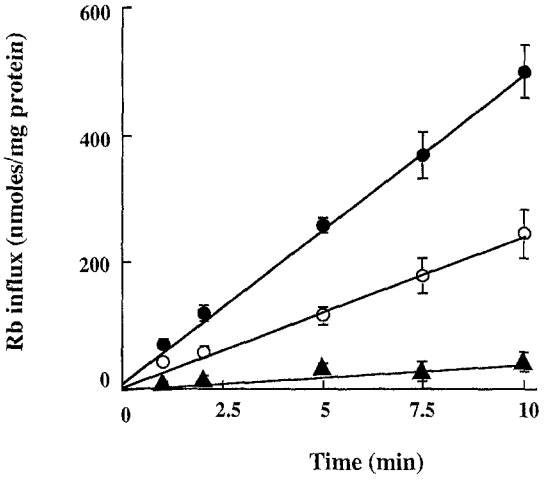

Fig. 1. Time course of the Rb influx into alveolar type II cells. ^{86}Rb influx was performed in the absence (filled circles) or presence of 1 mM ouabain (open circles) and ouabain plus 0.25 mM bumetanide (filled triangles). Data represent the mean ± SE of five experiments run in duplicate.

Table 1. Influence of various effectors on ^{86}Rb influx in alveolar type II cells

Inhibitors	^{86}Rb Influx (nmoles/mg prot/5 min)
Control	213.0 ± 11.4
+Ouabain (1 mM)	108.0 ± 9.5
+Bumetanide (0.25 mM)	166.0 ± 15.5
+Ouabain + bumetanide	34.3 ± 3.8
+Ouabain – Na^+free	44.1 ± 9.1
+Ouabain – Cl^- free	30.5 ± 6.7

Data represent the mean ± SE of 5 different experiments in which duplicates were obtained. ^{86}Rb influx was measured in alveolar epithelial cells in the absence (control) or presence of various inhibitors and Na^+ or Cl^- free medium as described in Materials and Methods.

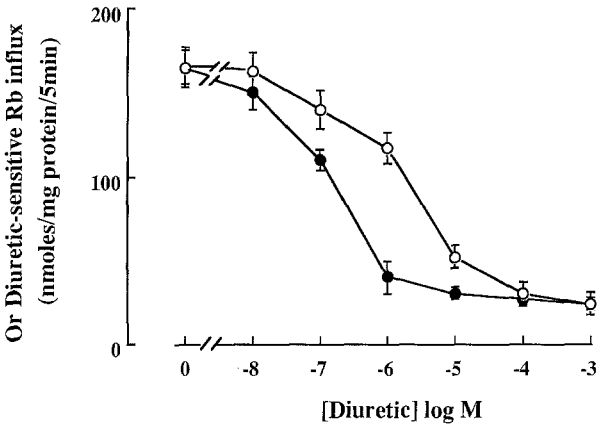

Fig. 2. Concentration-response inhibition of ouabain-resistant ^{86}Rb influx by furosemide and bumetanide in alveolar type II cells. Rb influx was measured in alveolar cells with 0.5 ml incubation medium containing ^{86}Rb (1 μCi/ml) plus 1 mM ouabain and increasing concentrations of furosemide (open circles) or bumetanide (filled circles). Data represent the mean ± SE of four experiments run in duplicate.

comprising 16% of total ^{86}Rb influx was unchanged by 1 mM $BaCl_2$, a channel K^+ blocker, suggesting that K^+ channels did not contribute to ^{86}Rb influx in alveolar type II cells (*data not shown*).

Dependency of the Ouabain-Resistant Bumetanide-Sensitive (Or-Bs) ^{86}Rb Influx on Extracellular K^+, Na^+ and Cl^-

To determine whether bumetanide-sensitive component of ^{86}Rb influx was consistent with a Na-K-Cl transport, we examined the extracellular K^+, Na^+ and Cl^- dependency of this component. ^{86}Rb influx was measured in the presence of ouabain in cells deprived of either Na^+ (isosmotically replaced by N-methyl-glucamine) or Cl^- (isosmotically replaced by gluconate) in the extracellular medium. Table 1 shows that removal of Na^+ or Cl^- from the incubation medium inhibited the ouabain-resistant ^{86}Rb influx with a pattern similar to that produced by bumetanide. The dependencies for extracellular Na^+ and K^+ were hyperbolic, with apparent Km of 14.6 ± 2.2, and 8.3 ± 1.4 mM respectively, with Hill coefficient of 1 (Fig. 3*A,B*). Figure 3*C* shows the extracellular Cl^- dependency of Or-Bs ^{86}Rb influx. This curve was sigmoidal with an apparent Km of 60.2 ± 1.34 mM and a Hill coefficient of 2.

Bumetanide-Sensitive ^{22}Na Influx

In the presence of extracellular K^+ and Cl^-, bumetanide decreased ^{22}Na influx, in alveolar type II cells, from 58 to 35 nmoles/mg protein/5 min (Fig. 4*A*). Although 1 mM amiloride produced a significant inhibition of ^{22}Na influx, bumetanide further reduced ^{22}Na influx, indicating that bumetanide-sensitive ^{22}Na influx pathway is distinct from the amiloride-sensitive ^{22}Na transport systems (Fig. 4*A*). The bumetanide-sensitive ^{22}Na influx was completely suppressed in the absence of either extracellular K^+ or Cl^- (Fig. 4*B*).

Effect of Time in Culture on Ouabain-Resistant Bumetanide-Sensitive ^{86}Rb Influx

^{86}Rb influx was studied on cultured alveolar cells from day 1 to day 4. The ouabain-resistant bumetanide-sensitive component of ^{86}Rb influx dramatically decreased with increasing time in culture (Fig. 5). At day 3 in culture and later, Or-Bs ^{86}Rb influx was decreased by more than 80%. In contrast, maintaining alveolar cells in culture did not affect Na,K-ATPase activity as measured by the ouabain-sensitive component of ^{86}Rb influx (*data not shown*).

Modulation of Ouabain-Resistant Bumetanide-Sensitive ^{86}Rb Influx by Agonists

Depending on the cell type or the animal species, the Na-K-Cl cotransporter has been reported to be regulated by second messenger pathways. As shown in Table 2, cyclic nucleotides did not affect the Na-K-Cl cotransport; Or-Bs ^{86}Rb influx was not modified by (i) 8 Br cGMP (8-bromoguanosine 3′,5′-cyclic monophosphate), (ii) 8 Br cAMP (8-bromoadenosine 3′,5′-cyclic monophosphate) or finally (iii) by stimulation of PKA through increased cAMP levels by forskolin or isoproterenol. In contrast, the phorbol ester, PMA, decreased Or-Bs ^{86}Rb influx. The inhibitory effect of PMA was time- and concentration-dependent and a maximal inhibition was observed for a concentration of 10^{-6} M using a preincubation of 30 min (Fig. 6). No effect of PMA was seen on ouabain-sensitive ^{86}Rb influx suggesting that the inhibitory effect of PMA is relatively specific for the Or-Bs ^{86}Rb influx. To determine the involvement of PKC in the phorbol ester-induced inhibition of Na-K-2Cl cotransport, we tested diacylglycerol analogues (OAG, diC8), which are physiological activators of PKC, as well as 4α-PDD, a phorbol ester which does not stimulate PKC. Table 3 shows that OAG and diC8 caused inhibition of bumetanide sensitive ^{86}Rb uptake whereas 4α-PDD was ineffective. A similar effect was observed in the presence of R59022, which prevents the conversion of DAG to phosphatidic acid (De Chaffoy de Courcelles, Roevens & Van Belle, 1985), and subsequently raises the intracellular DAG concentration. We also tested the specific inhibitor of PKC: staurosporine and showed that it completely prevented the inhibition of bumetanide-sensitive ^{86}Rb uptake by PMA.

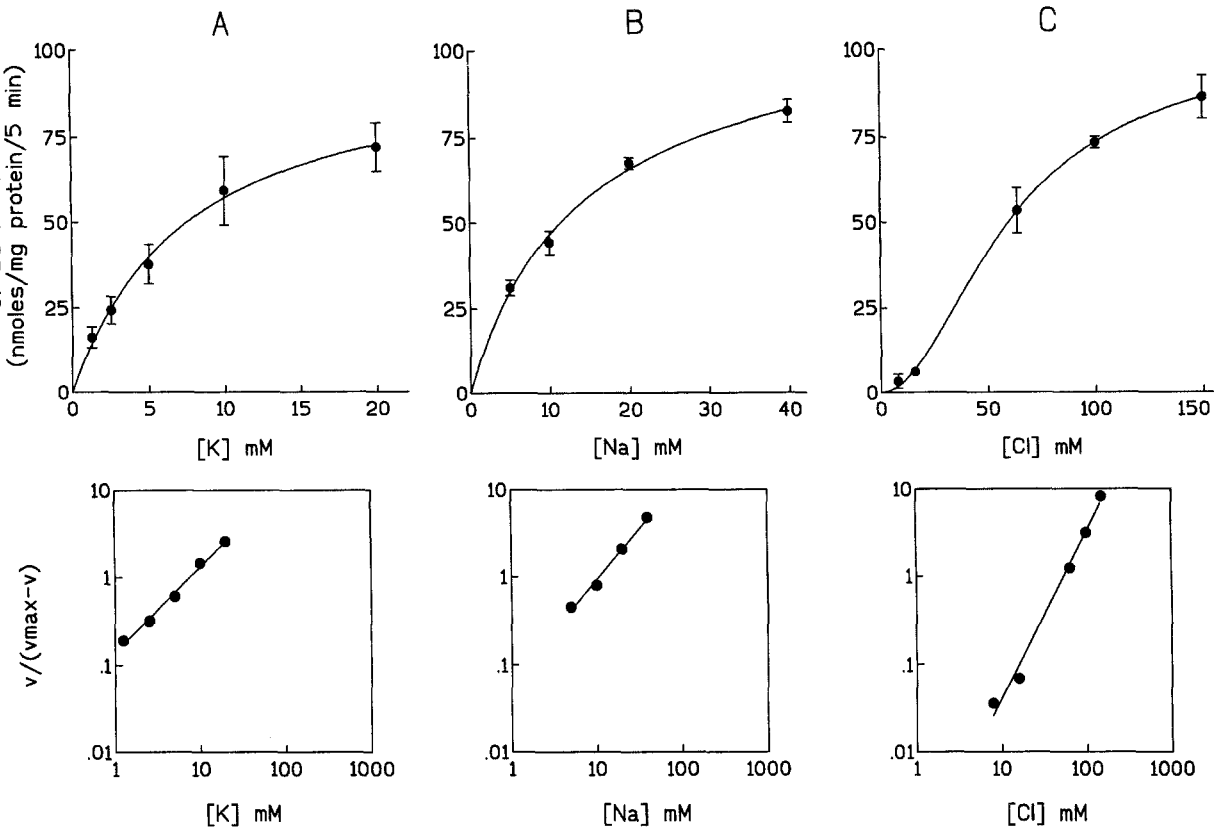

Fig. 3. Dependence of ^{86}Rb influx on extracellular ion concentrations. Upper panels: for each extracellular K^+ (*A*), Na^+ (*B*) and Cl^- (*C*) concentrations cells were incubated for 5 min with the indicated solution prior to being exposed to similar solutions containing ^{86}Rb, 1 mM ouabain with or without 0.25 mM bumetanide for 5 min. Lower panels: Hill plots calculated from mean values of the data shown in the upper panel. Data represent mean ± SE of three to four experiments run in duplicate.

Effect of Cell Volume Changes

The Na-K-Cl cotransporter is involved in cell volume regulation (Khademazad et al., 1991; Orlov et al., 1992). Therefore we investigated whether the Or-Bs ^{86}Rb influx in alveolar type II cells was affected by changes in extracellular osmolality. Figure 7 shows that exposure of the cells to a hypertonic solution had no effect on Or-Bs ^{86}Rb influx. However, if the cells were exposed to the swell-shrink procedure Or-Bs ^{86}Rb influx was markedly stimulated.

The interference between cell volume regulation and PKC activity was studied as described in Table 4. After inhibition with 200 ng/ml PMA prior to cell shrinking, Or-Bs sensitive ^{86}Rb influx could still be stimulated in shrunk cells. In that condition, the stimulation of Or-Bs ^{86}Rb influx had the same magnitude as in the control. In addition, when cells were depleted of PKC by incubation of cells with 100 ng/ml PMA for 24 hr, the magnitude of the stimulation of Or-Bs ^{86}Rb influx induced by cell swelling then shrinkage was unchanged.

Discussion

These data provide evidence for ^{86}Rb entry into alveolar type II cells via a ouabain-resistant bumetanide-sensitive pathway, which characteristics are consistent with a Na-K-Cl cotransport. This cotransport, which is modulated by intracellular mediators, may be involved in cell volume regulation.

Evidence for a Na-K-Cl Cotransport in Alveolar Type II Cells

In this study, several lines of evidence support the existence of a Na-K-Cl cotransport in alveolar type II cells. A fraction of total ^{86}Rb influx into alveolar type II cells was blocked by "loop diuretics," with a greater potency for bumetanide than for furosemide, as reported in a variety of tissues and cultured cells (O'Grady et al., 1987; Homma, Burns & Harris, 1990; Whisenant et al., 1991). This bumetanide-sensitive component of ^{86}Rb influx is clearly dependent on the presence of both extracellular Na^+ and Cl^- in the incubation medium. The stoichiometry of the Na-K-Cl cotransport system is generally thought to be 1-1-2 i.e., the system is electroneutral (O'Grady et al., 1987). Although we did not measure concomitant bumetanide-inhibitable influx of ^{22}Na, ^{86}Rb and ^{36}Cl, the alveolar type II cells cotransport system is likely to express the same ratio since the activation curve

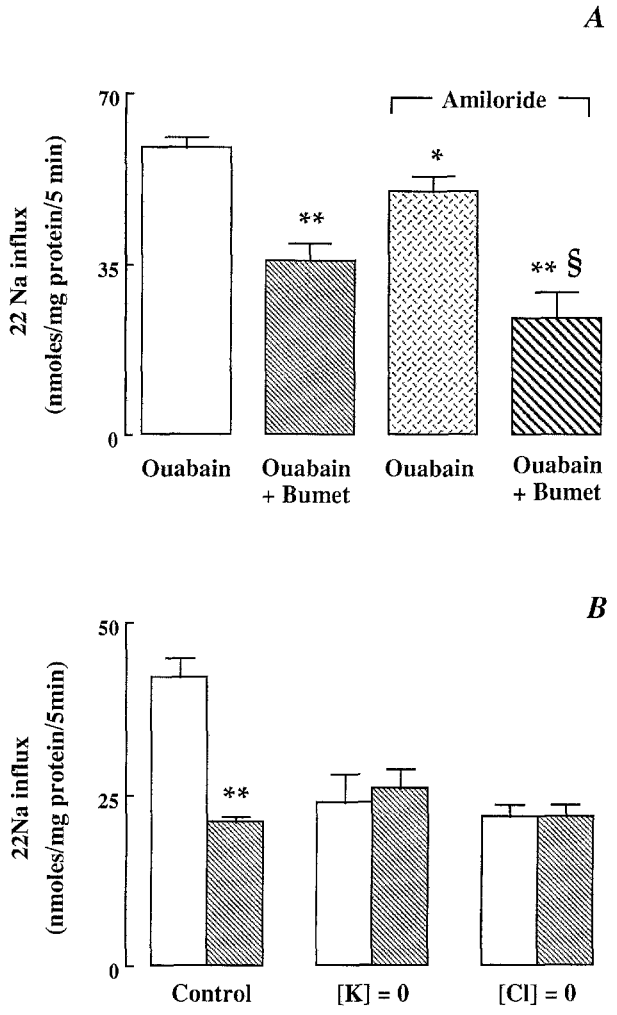

Fig. 4. Effects of transport inhibitors and extracellular Na^+ and Cl^- on ^{22}Na influx in alveolar type II cells. Cells were depleted in a Na^+-free N-methylglucamine (NMG) solution and ouabain was included to block efflux of ^{22}Na through Na,K-ATPase as described in Materials and Methods. (*A*) influx of ^{22}Na was measured in a solution consisting of (in mM) 14 NaCl, 35 KCl, and 96 NMG in the presence or absence of 0.25 mM bumetanide (Bumet) and 1 mM amiloride. (*B*) influx of ^{22}Na was measured in solutions consisting of (in mM) 14 NaCl and 131 NMG (K = 0) or of 14 Na gluconate, 35 K gluconate and 96 tetramethyl ammonium gluconate (Cl = 0) in presence (hatched bars) or in absence (open bars) of bumetanide (0.25 mM). Data represent the mean ± SE of three experiments run in duplicate. $*P < 0.05$, $**P < 0.01$ when compared with the control in presence of ouabain; $^{\S}P < 0.01$ when compared with the value in presence of ouabain plus amiloride.

for bumetanide-sensitive component of ^{86}Rb influx *vs.* extracellular Na^+ and K^+ concentrations were found to be simple hyperbolas, which suggests a single transport site, while the activation curve for bumetanide-sensitive component of ^{86}Rb influx *vs.* extracellular Cl^- concentration was sigmoid, which suggests more than one transport site. Care should be taken to interpret these data, however, since the shape of Cl^- activation curve was found to be dependent upon the substituting anion species employed (O'Grady et al., 1987; Owen & Prastein, 1985). Furthermore, gluconate may have chelated calcium, which might also affect the affinity of the cotransport system for Cl. At least, the presence of a Na-K-Cl cotransport was also supported by measurements of ^{22}Na influx: the amiloride-resistant ^{22}Na influx was inhibited by bumetanide, and the bumetanide-sensitive ^{22}Na influx required the presence of K^+ and Cl^- in the bath. It should be noticed that the bumetanide-sensitive uptake values we report for ^{22}Na and ^{86}Rb were not equal as would be expected for a 1-1-2 stoichiometry. However, these uptake studies were performed under dissimilar experimental conditions, the sodium concentration being lower in the case of the sodium uptake. Taken together, these data suggest the presence of a Na-K-2Cl cotransport in the plasma membrane of alveolar type II cells. The apparent Km for Na^+ and K^+, 14.6 mM and 8.3 mM, respectively, are close to the values reported for cell lines originating from distal nephron (O'Grady et al., 1987).

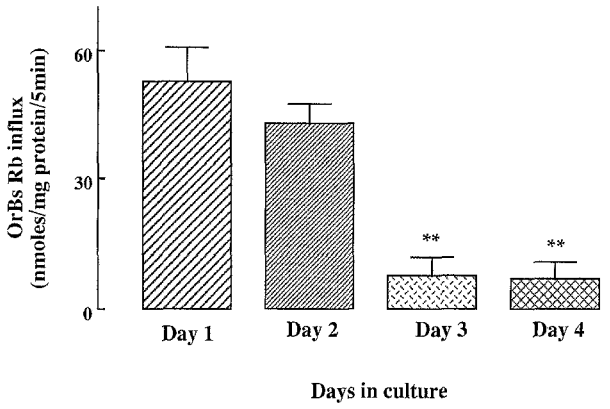

Fig. 5. Effect of time in culture on ouabain-resistant bumetanide-sensitive ^{86}Rb influx. Or-Bs ^{86}Rb influx was measured from day 1 to day 4 of the culture. Data represent the mean ± SE of four experiments run in duplicate. $**P < 0.01$ when compared with the value at day 1.

Table 2. Regulation by agonists and second messengers of Na-K-Cl activity in alveolar type II cells

Agonists	Or-Bs Rb influx (nmoles/mg protein/ 5 min)	*P*
Control	60.7 ± 6.0	
8 Br cAMP (10^{-4} M)	59.6 ± 11.0	ns
Forskolin (10^{-5} M)	51.1 ± 3.5	ns
Isoproterenol (10^{-5} M)	63.1 ± 10.1	ns
8 Br cGMP (10^{-5} M)	49.8 ± 7.8	ns
PMA (200 ng/ml)	29.5 ± 4.0	<0.01

Data represent the mean ± SE of 3–7 experiments performed in duplicate. Cells were incubated 30 min with the agonists prior to Or-Bs ^{86}Rb influx measurement. 8 Br AMPc, 8 bromo 3′, 5′adenosine monophosphate; 8 Br GMPc, 8 bromo guanosine monophosphate; PMA, phorbol myristate acetate. ns: not significantly different from the control value. <0.01: significantly different from the control value at $P < 0.01$.

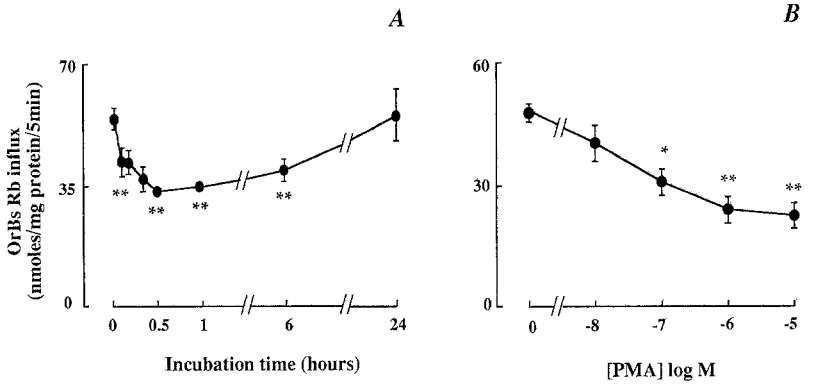

Fig. 6. Time and concentration-dependent inhibition of ouabain-resistant bumetanide-sensitive ^{86}Rb influx with PMA in alveolar type II cells. (*A*) time-dependent effects of PMA on Or-Bs ^{86}Rb influx. Cells were preincubated with 200 ng/ml PMA for the time periods indicated prior to determination of Or-Bs ^{86}Rb influx. (*B*) concentration-dependent effects of PMA. Cells were incubated for 30 min with the indicated concentrations of PMA prior to determination of Or-Bs ^{86}Rb influx. Data represent the mean ± SE of four experiments run in duplicate. $*P < 0.05$, $**P < 0.01$ when compared with the control.

Table 3. Effects of PMA analogues DAG kinase inhibitor, PMA inhibitor on Na-K-Cl cotransport activity in alveolar type II cells

Conditions	Or-Bs Rb influx (nmoles/mg protein/ 5 min)	*P*
Control	52.3 ± 4.9	
PMA (200 ng/ml)	25.5 ± 2.2	<0.01
PMA analogues		
OAG (50 μg/ml)	39.6 ± 3.7	<0.05
DiC8 (50 μg/ml)	37.4 ± 4.3	<0.05
4α-PDD (500 ng/ml)	45.0 ± 2.4	ns
DAG kinase inhibitor		
R59022 (1 μM)	29.1 ± 4.0	<0.01
PMA inhibitor		
Staurosporine (1 μM)	46.0 ± 7.6	ns
Staurosporine + PMA	45.1 ± 6.1	ns

Data represent the mean ± SE of 6 to 7 experiments run in duplicate. Cells were incubated 30 min with the agents prior to Or-Bs ^{86}Rb influx measurement. ns: not significantly different from the control value. <0.05, <0.01: significantly different from the control value at $P < 0.05$ and $P < 0.01$.

Previous studies on the transport properties of alveolar type II cells brought controversial results about the presence of a Na-K-Cl cotransport. In rat alveolar type II cells grown on porous supports, furosemide when applied on either the apical or the basolateral side did not change the transepithelial potential difference after 6 days of culture (Mason et al., 1982; Cott, Sughara & Mason, 1986). Similarly in a recent study, Russo et al. (1992) reported that ^{22}Na uptake in rat alveolar epithelial cells, cultured on plastic cover slips for 3–5 days, was unaffected by furosemide. In the contrary, Bland and Boyd (1986) demonstrated in freshly isolated alveolar cells from rabbit the presence of a furosemide-sensitive component of ^{86}Rb influx, consistent with the presence of a Na-K-Cl cotransport. These discrepancies could be ascribed to animal species or experimental models (freshly isolated *vs.* cultured cells). However, the present study demonstrates the presence of a Na-K-Cl cotransport in alveolar type II cells from adult rat. Furthermore, the activity of the cotransport dramatically declined after 48 hr in culture, which might explain the previous negative reports (Mason et al., 1982; Cott et al., 1986; Russo et al., 1992). It is well established that alveolar type II cells phenotype changes in culture: surfactant secretion decreases, cell surface binding characteristics change, and activity of Na-dependent phosphate transport drops (Dobbs, 1990; Clerici, Soler & Saumon, 1991). The decrease in Na-K-Cl cotransport paralleled these changes and thus may be due either to culture-induced dedifferentiation or to transformation of these cells into alveolar type I cells. As compared with other absorptive epithelia, this Na-K-Cl cotransport would be expected to be present on the apical membrane of the cells. However, we were not able to determine the location of the Na-K-Cl cotransport in alveolar epithelial cells, since in our conditions cell polarization required several days after seeding for spreading and formation of junctional complexes (*unpublished results*), a delay during which the activity of the cotransport vanishes.

The Na-K-Cl Cotransport is Modulated by PKC, but not by cAMP or cGMP

Depending on the animal species, activity of the Na-K-Cl cotransport is modulated by various factors such as intracellular cAMP content (Whisenant et al., 1991; Vuillemin et al., 1992), intracellular cGMP content (O'Grady et al., 1985; O'Donnell & Owen, 1986), intracellular calcium (Homma, Hoover & Harris, 1990*b*) or activity of PKC (O'Brien & Krzeminski, 1983; Franklin, Turner & Kim, 1989; Crook, Von Brauchitsch & Polansky, 1992). We report that the phorbol ester PMA inhibited the Na-K-Cl cotransport in alveolar type II cells, which was affected neither by cAMP nor by cGMP level. This PMA-induced inhibition was related to specific activation of PKC since: (i) 4α-PDD, a phorbol ester ineffective to stimulate PKC, did not affect cotransport activity; (ii) the effect of PMA was mimicked by two permeant diacylglycerol analogues, OAG and DiC8, at concentrations reported to stimulate PKC in several cell

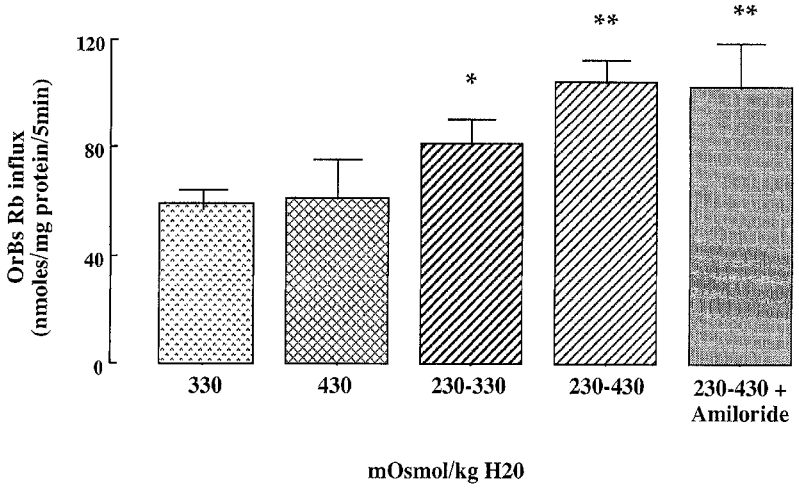

Fig. 7. Effect of cell volume changes on ouabain-resistant bumetanide-sensitive ^{86}Rb influx in alveolar type II cells. Cells were incubated with either isotonic solution (330 mOsm/kgH_2O) or hypertonic medium (430 mOsm/kgH_2O), similar to isotonic solution but with addition of sucrose, for 30 min prior to Or-Bs ^{86}Rb influx measurement. In other experiments, cells were first exposed to a hypotonic solution (230 mOsm/kgH_2O), similar to *solution A* but with only 70 mM NaCl, for 15 min prior to being exposed to isotonic (230–330 mOsm/kgH_2O) or hypertonic (230–430 mOsm/kgH_2O) for 15 min, then Or-Bs ^{86}Rb influx was measured. Data represent the mean ± SE of four to six experiments run in duplicate. $*P <$ 0.05, $**P < 0.01$ when compared with the isotonic control (330 mOsm/kgH_2O).

Table 4. Relationship between different modes of Na-K-Cl regulation in alveolar type II cells

Conditions	Or-Bs Rb influx (nmoles/mg protein/ 5 min)	(% of control)
Control (330 mOsm/kgH_2O)	46.8 ± 6.6	(100)
Swell then shrink (230 then 430 mOsm/kgH_2O)	82.4 ± 8.4	(180)
PKC activation		
Control isotonic with PMA (15 min)	22.0 ± 2.0	(100)
Swell then shrink with PMA (15 min)	41.1 ± 3.1	(185)
PKC downregulated		
Control isotonic (PMA 24 hr)	52.8 ± 5.4	(100)
Swell then shrink (PMA 24 hr)	85.0 ± 6.3	(163)

Data represent the mean ± SE of 3 to 5 experiments performed in duplicate. Cells were incubated in either isotonic medium (330 mOsm/kgH_2O) (control) or hypotonic medium (230 mOsm/kgH_2O) for 15 min followed by exposure to hypertonic medium (430 mOsm/kgH_2O) for 15 min (swell then shrink) as described in Materials and Methods. PKC inhibition was obtained by incubating the cells with 200 ng/ml PMA during hypertonic exposition; PKC was downregulated by incubating the cells with 100 ng/ml PMA for 24 hr prior to manipulations of extracellular osmolality.

types; (iii) the effect of PMA, OAG and DiC8 was mimicked by R59022, a DAG kinase inhibitor, which elevates the cellular DAG content (De Chaffoy de Courcelles et al., 1985); and (iiii) the inhibitory effect of PMA was reversed by staurosporine, an inhibitor of PKC. In alveolar type II cells, PMA treatment caused a rapid and sustained decrease of Na-K-Cl cotransport activity, followed by a recovery at 24 hr. This kinetics emphasizes the direct inhibitory role of PKC, since long-term treatment with PMA (>6 hr), which downregulates PKC activity (Franklin et al., 1989), resulted in our conditions in a recovery of the cotransport activity. The inhibition of Na-K-Cl cotransport by phorbol esters in alveolar type II cells is similar to the effect observed in other epithelial cells such as intestinal cells (Franklin et al., 1989) and ocular ciliary body (Crook et al., 1992), as well as in nonepithelial cells such as vascular smooth muscle (Owen, 1985) and osteoblasts (Whisenant et al., 1991). The mechanisms involved in the PKC-dependent inhibition of Na-K-Cl activity are unclear. A decrease in the number of Na-K-Cl cotransports was demonstrated in PMA-stimulated HT29 cells (Franklin et al., 1989), but this process was not characterized, i.e., direct phosphorylation of the cotransport, *vs.* interaction with a cytoplasmic phosphorylated unknown protein.

The Cotransport is Activated in Alveolar Type II Cells by Cell Volume Changes

In epithelial as well as nonepithelial cells, Na-K-Cl cotransport is involved in cell volume regulation (Khademazad et al., 1991; Orlov et al., 1992). Activation of Na-K-Cl cotransport is one of the mechanisms which allow cells to recover from shrinking, a process called regulatory volume increase (Strange, 1994). In the case of alveolar type II cells studied under basal conditions, shrinking did not change Na-K-Cl cotransport activity. However, activation of the Na-K-Cl cotransport activity was observed when cells were first swelled in hypotonic medium prior to shrinking, a protocol that has been used for other cell types (Hoffmann, Sjoholm & Simonsen, 1983; Whisenant et al., 1991). In the first situation, shrinking the cells increases intracellular ion concentrations and thus reduces the driving force for the cotransport, which might be further inhibited by intracellular Cl^- acting on a regulatory site, as shown in the squid axon (Breitwieser, Altamirono & Russell, 1990). In the latter situation, exposure of cells to hypotonic medium results in a loss of K^+ and Cl^- during regulatory volume

decrease (Strange, 1994). Then, K^+ and Cl^- transmembrane gradients will be increased during subsequent exposure to isotonic or hypertonic solution, which may increase the cotransport activity. This process is most probably involved in the stimulation of Na-K-Cl cotransport by the swell- and shrink-procedure.

To determine whether protein kinase C is the prominent transducer of the modulation of Na-K-Cl cotransport activity in response to changes in cell volume, the swell- and shrink-procedure was performed on cells in which PKC was either activated or downregulated. Cotransport activity was similarly increased by cell shrinkage in control as well as in PKC-stimulated or downregulated cells. These results indicate that the cotransport activity might be modulated through different independent pathways. In fact, modulation of cotransport activity by cyclic AMP, Ca-calmodulin and PKC was shown to be unrelated to the regulation of cell volume (Whisenant et al., 1991; Orlov et al., 1992).

In summary, we report the presence of a Na-K-Cl cotransport in cultured adult rat alveolar type II cells. Strong evidence suggests that this cotransporter is modulated by the level of protein kinase C activity and is activated by cell volume change. Interestingly, the different modulators, i.e., cell volume change or protein kinase C activity, appear to act independently to modify cotransport activity. Though we were not able to determine the polarity of the cotransport, it may also be involved in the absorptive function of alveolar type II cells, and would provide an alternate pathway for sodium entry.

This work was supported by grants from INSERM, CNRS, Université Denis Diderot Paris 7, Faculté Xavier Bichat, Fondation pour la Recherche Médicale, and Laboratoire de Recherches Physiologiques.

References

Basset, G., Crone, C., Saumon, G. 1987. Fluid absorption by rat lung in situ: pathways for sodium entry in the luminal membrane of alveolar epithelium. *J. Physiol. Lond.* **384:**325–345

Bland, R.D., Boyd, C.A.R. 1986. Cation transport in lung epithelial cells derived from fetal, newborn, and adult rabbits. *J. Appl. Physiol.* **61:**507–512

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* **72:**248–254

Breitwieser, G.E., Altamirano, A.A., Russell, J.H. 1990. Osmotic stimulation of Na,K,Cl cotransport in squid giant axon is [Cl]i dependent. *Am. J. Physiol.* **258:**C749–C753

Cheval, L., Doucet, A. 1990. Measurement of Na-K-ATPase-mediated rubidium influx in single segments of rat nephron. *Am. J. Physiol.* **259:**F111–F121

Clerici, C., Friedlander, G., Amiel, C. 1992. Impairment of sodium-coupled uptakes by hydrogen peroxide in alveolar type II cells: protective effect of d-α-tocopherol. *Am. J. Physiol.* **262:**L542–L548

Clerici, C., Soler, P., Saumon, G. 1991. Sodium-dependent phosphate and alanine transports but sodium-independent hexose transport in type II alveolar cells in primary culture. *Biochim. Biophys. Acta* **1063:**27–35

Cott, G.R., Sughara, K., Mason, R. 1986. Stimulation of net active ion transport across alveolar type II cell monolayers. *Am. J. Physiol.* **250:**C222–C227

Crook, R.B., Von Brauchitsch, D.K., Polansky, J.R. 1992. Potassium transport in nonpigmented epithelial cells of ocular ciliary body: inhibition of Na^+, K^+, Cl^- cotransporter by protein kinase C. *J. Cell Physiol.* **153:**214–220

De Chaffoy de Courcelles, D., Roevens, P., Van Belle, H. 1985. R 59 022, a Diacylglycerol Kinase Inhibitor. *J. Biol. Chem.* **260:**15762–15770

Dobbs, L.G. 1990. Isolation and culture of alveolar type II cells. *Am. J. Physiol.* **258:**L134–L147

Franklin, C.C., Turner, J.T., Kim, H.D. 1989. Regulation of Na/K/Cl cotransport and [3H] bumetanide binding site density by phorbol esters in HT29 cells. *J. Biol. Chem.* **264:**6667–6673

Goodman, B.E., Crandall, E.D. 1982. Dome formation in primary cultured monolayers of alveolar epithelial cells. *Am. J. Physiol.* **243:**C96–C100

Hoffmann, E.K., Sjoholm, C., Simonsen, L.O. 1983. Na, Cl cotransport in Ehrlich ascites tumor cells activated during volume regulation (regulatory volume increase). *J. Membrane Biol.* **76:**269–280

Homma, T., Burns, K.D., Harris, R.C. 1990*a*. Agonist stimulation of Na/K/Cl cotransport in rat glomerular mesangial cells. *J. Biol. Chem.* **265:**17613–17620

Homma, T., Hoover, R.L., Harris, R.C. 1990*b*. Loop diuretic-sensitive potassium flux pathways of rat glomerular mesangial cells. *Am. J. Physiol.* **258:**C862–C870

Khademazad, M., Zhang, B.X., Loessberg, P., Muallem, S. 1991. Regulation of cell volume by the osteosarcoma cell line UMR-106-01. *Am. J. Physiol.* **261:**C441–C447

Mason, R.J., Williams, M.C., Widdicombe, J.H., Sanders, M.J., Misfeld, D.S., Berry, L.C. 1982. Transepithelial transport by pulmonary alveolar type II cells in primary culture. *Proc. Natl. Acad. Sci. USA* **79:**6033–6037

Matalon, S. 1991. Mechanisms and regulation of ion transport in adult mammalian alveolar type II pneumocytes. *Am. J. Physiol.* **261:**C727–C738

Matalon, S., Bridges, R.J., Benos, D.J. 1991. Amiloride-inhibitable Na conductive pathways in alveolar type II pneumocytes. *Am. J. Physiol.* **260:**L90–L96

O'Brien, T.G., Krzeminski, K. 1983. Phorbol ester inhibits furosemide-sensitive potassium transport in BALB/c 3T3 preadipose cells. *Proc. Natl. Acad. Sci. USA* **80:**4334–4338

O'Donnell, M.E., Owen, N.E. 1986. Atrial natriuretic factor stimulates Na/K/Cl cotransport in vascular smooth muscle cells. *Proc. Natl. Acad. Sci. USA* **83:**6132–6136

O'Grady, S.M., Field, M., Nash, N.T., Rao, M.C. 1985. Atrial natriuretic factor inhibits Na-K-Cl cotransport in teleost intestine. *Am. J. Physiol.* **249:**C531–C534

O'Grady, S.M., Palfrey, H.C., Field, M. 1987. Characteristics and function of Na-K-2Cl cotransport in epithelial tissues. *Am. J. Physiol.* **253:**C177–C192

Orlov, S.N., Resink, T.J., Bernhardt, J., Bulher, F. 1992. Volume-dependent regulation of sodium and potassium fluxes in cultured vascular smooth muscle cells: dependence on medium osmolality and regulation by signalling systems. *J. Membrane Biol.* **129:**199–210

Owen, N.E. 1985. Effect of TPA on ion fluxes and DNA synthesis in vascular smooth muscle cells. *J. Cell Biol.* **101:**454–459

Owen, N.E., Prastein, M.L. 1985. Na/K/Cl cotransport in cultured human fibroblasts. *J. Biol. Chem.* **260:**1445–1451

Rotin, D., Grinstein, S. 1989. Impaired cell volume regulation in Na-H exchange-deficient mutants. *Am. J. Physiol.* **257:**C1158–C1165

Russo, R.M., Lubman, R., Crandall, E.D. 1992. Evidence for amiloride-sensitive sodium channels in alveolar epithelial cells. *Am. J. Physiol.* **262:**L405–L411

Strange, K. 1994. Are all cell volumes changes the same? *News Physiol. Sci.* **9:**223–228

Vuillemin, T., Teulon, J., Geniteau-Legendre, M., Baudouin, B., Estrade, S., Cassingena, R., Ronco, P., Vandewalle, A. 1992. Regulation by calcitonin of Na-K-Cl cotransport in a rabbit thick ascending limb cell line. *Am. J. Physiol.* **263:**C563–C572

Whisenant, N., Zhang, B.X., Khademazad, M., Loessberg, P., Muallem, S. 1991. Regulation of Na-K-2Cl cotransport in osteoblasts. *Am. J. Physiol.* **261:**C433–C440