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SECOND-MESSENGER REGULATION OF SODIUM TRANSPORT IN MAMMALIAN AIRWAY EPITHELIA

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SUMMARY

1. Sodium absorption is the dominant ion transport process in conducting airways and is a major factor regulating the composition of airway surface liquid. However, little is known about the control of airway sodium transport by intracellular regulatory pathways.

2. In sheep tracheae and human bronchi mounted in Ussing chambers under short circuit conditions, the sodium current can be isolated by pretreating tissues with acetazolamide (100 μM) to inhibit bicarbonate secretion, bumetanide (100 μM) to inhibit chloride secretion and phloridzin (200 μM) to inhibit sodium–glucose co-transport. This sodium current consists of amiloride-sensitive (57 %) and amiloride-insensitive (43 %) components.

3. The regulation of the isolated sodium current by three second messenger pathways was studied using the calcium ionophore A23187 to elevate intracellular calcium, a combination of forskolin and the phosphodiesterase inhibitor zardaverine to elevate intracellular cyclic AMP, and the phorbol ester 12,13-phorbol dibutyrate (PDB) to stimulate protein kinase C.

4. In sheep trachea, A23187 produces a dose-related inhibition of the sodium current with maximal effect (38 % of I_{sc}) at 10 μM and IC_{50} 1 μM. This response affects both the amiloride-sensitive and insensitive components of the sodium current and is not altered by prior stimulation of protein kinase C or elevation of intracellular cyclic AMP. In human bronchi, A23187 (10 μM) produced a significantly greater inhibition of I_{sc} (68 %), a response which was unaffected by prior treatment with PDB or forskolin–zardaverine.

5. In sheep trachea, stimulation of protein kinase C with PDB produced a dose-related inhibition of I_{sc} maximal (56 % of I_{sc}) at 50 nM (IC_{50} 7 nM). This response was abolished by amiloride (100 μM) pretreatment suggesting a selective effect on the amiloride-sensitive component of the sodium current. The response was not altered by prior elevation of intracellular calcium or cyclic AMP. PDB (10 nM) caused a similar inhibition of I_{sc} in human bronchi (43 %). The effect of PKC stimulation following pretreatment with A23187 was diminished in human bronchi. Elevating intracellular cyclic AMP did not alter this response.

6. Addition of forskolin (1 μM) together with the phosphodiesterase inhibitor zardaverine (100 μM) produced a mean 35-fold increase in intracellular cyclic AMP in

sheep trachea. This was associated with a small, but significant, 6% transient increase in I_{sc} followed by a significant 4% fall. Neither effect could be abolished by amiloride pretreatment. In human bronchi, a small decrease in I_{sc} which could not be distinguished from that occurring in controls was observed.

7. We conclude that elevating intracellular calcium and protein kinase C stimulation have marked inhibitory effects on sodium transport in sheep trachea and human bronchus. Stimulation of protein kinase A does not appear to have a major role in the regulation of sodium absorption in these tissues.

INTRODUCTION

Airway epithelial ion transport is a major factor determining the composition of airway surface liquid. In several species, including man, the dominant ion transport process in conducting airways is sodium absorption (Welsh, 1987). This is accompanied by absorption of fluid from the airway surface liquid (ASL), probably through the intercellular spaces, along the osmotic gradient created (Nathanson, Widdicombe & Nadel, 1983; Widdicombe, Gashi, Basbaum & Nathanson, 1985). The hydration of the liquid overlying airway epithelia is likely to influence mucociliary clearance (MCC). Thus, in cystic fibrosis (CF), in which airway sodium absorption is increased 2- to 3-fold (Boucher, Cotton, Gatzy, Knowles & Yankaskas, 1988), MCC can be improved with nebulized amiloride *in vivo* (App, King, Helfesreider, Köhler & Matthys, 1990). Absorption of sodium and water across conducting airways may also be involved in the resolution of pulmonary oedema, although the relative contribution, if any, of these sites compared to the alveolar epithelium which occupies a much greater proportion of the pulmonary surface area is unknown (O'Brodovich, 1990).

While much attention has been focused on the regulation of chloride secretion by neurohumoral factors and intracellular messengers with particular reference to CF, far less is known about the regulation of sodium absorption in conducting airways. Unlike chloride impermeability, which is a feature of many tissues in CF, increased sodium absorption appears to be unique to airways. However, it quantitatively dominates ion transport at this principal site of morbidity and mortality in this disease. Furthermore, if sodium absorption is an important factor affecting MCC, then an understanding of its regulation may also be of relevance to other diseases in which MCC is impaired.

One problem in the study of airway sodium transport regulation has been the lack of a suitable model in which to study the sodium current in isolation. Without such isolation, induction of other ion transport processes such as chloride secretion by second messengers may not only obscure any effect on the sodium current, but may lead to secondary changes in it. In previous ion transport characterization studies it has not been possible to account for the total composition of the short circuit current (I_{sc}), an unexplained residual current of up to 30% remaining in addition to the measured sodium and chloride fluxes (Cotton, Lawson, Boucher & Gatzy, 1983; Jarnigan, Davis, Bromberg, Gatzy & Boucher, 1983; Knowles, Murray, Shallal, Askin, Ranga, Gatzy & Boucher, 1984). The only previous approach to the study of regulation of sodium transport in conducting airways involved removal of chloride from the solution bathing the tissue (Cullen & Welsh, 1987). However, this

manoeuvre may reduce sodium absorption by removing its associated anion, and may not exclude the contribution of other, unidentified, ion transport processes.

We have recently characterized the components of the I_{sc} in two sodium-absorbing epithelia, sheep trachea and human bronchus (Steel, Graham, Alton & Geddes, 1991*a*). Both tissues showed a similar pattern, with bicarbonate and chloride secretion and sodium–glucose co-transport contributing approximately 30% of the total I_{sc}, as evidenced by inhibition of these processes with acetazolamide, bumetanide and phloridzin respectively. The remaining current could be completely abolished by sodium substitution in the mucosal bathing solution. This sodium-dependent portion of the current showed amiloride-sensitive (57%) and amiloride-insensitive (43%) components and its magnitude was independent of the presence of the other ion transport processes discussed above. It is subsequently referred to as the 'isolated sodium current'.

In this study we have used sheep and human (non-CF) airway epithelia mounted in Ussing chambers under short circuit conditions to investigate the regulation of this isolated sodium current by three major intracellular pathways. Intracellular calcium was elevated using the calcium ionophore A23187, protein kinase C (PKC) stimulated using the phorbol ester 12,13-phorbol dibutyrate (PDB) and intracellular cyclic AMP levels were elevated using a combination of forskolin and the type 3 and 4 phosphodiesterase inhibitor zardaverine (Schudt, Winder, Muller & Ukena, 1991). Intracellular calcium and protein kinases A and C are all known to have effects on airway epithelial chloride transport (Frizzell, Halm, Rechkemmer & Shoemaker, 1986; Li, McCann, Anderson, Clancy, Liedtke, Nairn, Greengard & Welsh, 1989) and on sodium transport in other epithelia (Garty & Benos, 1988; Turnheim, 1991). The effects of both single and sequential addition of these agents were studied and comparisons made between responses in sheep tracheae and human bronchi.

METHODS

Ussing chamber studies

Sheep trachea were obtained fresh from a local abattoir and immersed immediately in cold (4 °C) Krebs–Henseleit solution of composition (mM): Na^+, 145·0; Cl^-, 126·0; K^+, 5·9; Ca^{2+}, 2·5; Mg^{2+}, 1·2; HCO_3^-, 26·0; PO_4^{2-}, 1·2; SO_4^{2-}, 1·2; glucose, 5·6. The epithelium was dissected free of underlying tissues and mounted in Ussing chambers of area 1·28 cm^2. Human bronchi were obtained from whole diseased lungs (hypogammaglobulinaemia, primary pulmonary hypertension and Eisenmenger syndrome (2 lungs)) removed at transplantation and also immersed immediately in cold Krebs–Henseleit solution. For the main bronchi the epithelium was partly dissected from underlying tissues, but the smaller bronchi were simply opened longitudinally before mounting. Both were mounted in Ussing chambers of area 0·28 cm^2. All tissues were bathed with Krebs–Henseleit solution at 37 °C (pH 7·4) and bubbled through gas lifts with a 95% O_2–5% CO_2 mixture. The potential difference (PD) across the tissue was measured using 1 M-KCl–2% agar bridges connected to a DVC-1000 voltage clamp (World Precision Instruments) by calomel electrodes (Russell pH Limited). The offset of the electrodes was measured prior to tissue mounting and suitable adjustments made to recorded values. Short circuit current (I_{sc}) was measured using 0·9% NaCl–2% agar bridges connected to the clamp by Ag–AgCl electrodes. The tissues were maintained continuously under short circuit conditions except for brief (15 s) intervals to measure PD. Conductance (g) was calculated from Ohm's law. Tissues with a conductance of greater than 10 mS cm^{-2} (sheep trachea) and 14 mS cm^{-2} (human bronchus) (Knowles *et al.* 1984) were excluded. Once tissues were electrically stable, defined by a rate of rise in I_{sc} of less than 3 $\mu A\ h^{-1}$ (sheep trachea) and 0·4 $\mu A\ h^{-1}$ (human bronchus), experimental interventions were commenced.

Experimental protocols

To isolate the sodium current tissues were pretreated sequentially with serosal acetazolamide (100 μM) for 20 min, serosal bumetanide (100 μM) for 10 min and mucosal phloridzin (200 μM) for 10 min (Steel *et al.* 1991*a*). Longer periods of time (up to 60 min) were required for serosally applied drugs to exert their maximal effect in human bronchi, as the serosal surface had not been fully cleared of underlying tissues. In experiments in which tissues were also pretreated with amiloride (100 μM), this was added mucosally for 5 min prior to further intervention. In experiments in which the putative PKC inhibitor calphostin C (Koybayashi, Nakano, Morimoto & Tamaoki, 1989) was used, this was added mucosally for 30 min before addition of PDB.

For controls, the sodium current was isolated as for the tissues receiving an intervention and tissues then treated with diluent, except in the case of PDB where equivalent concentrations (10 nM) of the stereoisomer 4α-PDB were used. In experiments using human bronchi a progressive fall in I_{sc} occurred, unlike sheep trachea where the I_{sc} remained stable for the time course of our experiments. A completely untreated control was therefore also used to compare this fall with the effects of diluent. When drugs were added sequentially, each was left to exert its full effect before addition of the next agent. Tissues were paired from the same animal unless otherwise indicated.

Measurement of intracellular cyclic AMP levels

On removal from the Ussing chambers the portion of tracheal epithelium which had been exposed to the bathing solutions was detached from the surrounding tissue, snap frozen in liquid nitrogen, and stored at −80 °C until assayed. Standard methods for measurement of cyclic AMP levels were used (Brooker, Harper, Kawasaki & Moylan, 1979). Briefly, tissues were weighed, homogenized in 6% trichloracetic acid and centrifuged at 25000 g_{av} at 4 °C for 15 min. The supernatant was added to 10 mM-EDTA and a freon:octylamine mixture added to extract cyclic nucleotides. Samples were spun in a microfuge at 12000 g_{av} for 5 min after which the upper aqueous phase was removed and neutralized with 60 mM-$NaHCO_3$. Samples were then diluted with 50 mM-sodium acetate buffer and acetylated by the sequential addition of triethylamine and acetic anhydride. ^{125}I-cyclic AMP and cyclic AMP antibody were added to each acetylated sample and these were then incubated overnight at 4 °C. Ice-cold potassium phosphate charcoal suspension was added to each sample which was left for 10 min before being microfuged at 12000 g_{av} for 2 min. Aliquots (600 μl) of the resulting supernatant were counted in a gamma counter, and the cyclic AMP in each sample determined by extrapolation from a standard curve.

Preparation and assay of C kinase from sheep tracheal epithelial cells

The epithelial cell layer from sheep tracheae was homogenized (Polytron PT 10) for 2 × 15 s bursts in 10 vol (w/v) of ice-cold Buffer A containing (mM): MOPS, 10 (pH 7·2); EDTA, 2; EGTA, 5; DTT, 10; PMSF, 0·1; leupeptin, 0·1; followed by centrifugation for 20 min at 45000 g_{av} at 4 °C to form soluble (supernatant) and particulate (pellet) fractions. The supernatant was applied to a column of Q-sepharose pre-equilibrated in Buffer A, and C-kinase was eluted with 10 ml of Buffer A with 130 mM-KCl.

C-kinase activity was measured in the presence and absence of calphostin C (10 μM) using a modification (Cook, Neville, Vrana, Hartl & Roskoski, 1982) of the method of Witt & Roskoski (1975). Briefly, C-kinase was assayed in triplicate at 30 °C and the reaction was initiated by the addition of 25 μM of the post Q-sepharose extract to 75 μl of a reaction medium (pH 7·2) containing: MOPS, 20 mM; magnesium acetate, 15 mM; ATP, 0·1 mM supplemented with 100 c.p.m. per pmole [δ-^{32}P]ATP (20–40 Ci mmol^{-1}); $CaCl_2$, 1·5 mM, PDB, 500 nM; phosphatidylserine, 100 μg ml^{-1}; histone IIIs, 1 mg ml^{-1}; BSA, 2 mg ml^{-1}; A-kinase inhibitor peptide (Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp), 1 μM. The reaction was terminated after 10 min by pipetting 75 μM aliquots of the mixture onto 2 cm × 2 cm phosphocellulose paper squares (Whatman P81) which were left for 30 s and then immersed in phosphoric acid (150 mM). The paper squares were extensively washed (4 × 5 min) in fresh phosphoric acid to remove any non-specifically bound labelled ATP and inorganic phosphate, immersed in absolute alcohol (5 min) and diethylether (5 min) and allowed to dry. The bound radioactivity representing *phospho*histone IIIs was quantified by liquid scintillation spectrometry in 4 ml ACS II. Protein was estimated using BSA (bovine serum albumin) as standard (Lowry, Rosebrough, Farr & Randall, 1951).

Drugs and chemicals

4α-12,13-Phorbol dibutyrate was obtained from LC Services Corporation and acetazolamide from Lederle Laboratories. Amiloride was a gift of Merck, Sharp and Dohme and zardaverine a gift of Dr C. Schutd. Calphostin C was obtained from Kamiya Medical Company. Trioctylamine and 1,1,2-trichlorotrifluoroethane were obtained from Aldrich Chemical Company and all other chemicals were obtained from Sigma Chemical Company. For Ussing chamber studies, A23187, zardaverine, and calphostin C were dissolved in DMSO (dimethyl sulphoxide) and added to tissues in dilutions of 1 in 1000. PDB, 4α-PDB and bumetanide were dissolved in ethanol and added to tissues in a dilution of 1 in 1000. Forskolin was dissolved in ethanol followed by serial dilutions in Krebs–Henseleit solution to give a final forskolin concentration of 100 μM in a 1 in 10^5 Krebs:ethanol mixture, which was added to tissues in a dilution of 1 in 100. Amiloride, phloridzin and acetazolamide were all dissolved in Krebs–Henseleit solution at 37 °C and added in dilutions of 1 in 10, 1 in 50 and 1 in 100 respectively.

Statistics

The Mann–Whitney *U* test was used to compare means. The Wilcoxon rank sum test was used to compare the effects of an intervention against baseline. Results are expressed as mean (S.E.M.) for convenience.

RESULTS

Baseline bioelectric properties for sheep tracheal epithelium ($n = 225$ tissues, 57 sheep) were: PD = 11·1 (0·3) mV, I_{sc} = 58 (2) μA cm^{-2} and g = 5·6 (0·1) mS cm^{-2}. For human bronchial epithelium ($n = 27$ tissues, 4 individuals) corresponding values were: PD = 2·1 (0·2) mV, I_{sc} = 23 (2) μA cm^{-2} and g = 11·6 (0·7) mS cm^{-2}.

Effects of tissue pretreatment protocols

The effects of tissue pretreatment with serosal acetazolamide (100 μM), serosal bumetanide (100 μM) and mucosal phloridzin (200 μM) in sheep tracheae and human bronchi are summarized in Table 1. The mean isolated sodium current of 40 (1·0) μA cm^{-2}, accounting for 69% of the total I_{sc} in sheep tracheae, and of 17 (2) μA cm^{-2}, accounting for 74% of the total I_{sc} in non-CF human bronchi, are in keeping with the results of our previous study (Steel *et al.* 1991*a*). Further addition of amiloride (100 μM) ($n = 12$) in sheep tracheae resulted in a fall in I_{sc} of 20 (4) μA cm^{-2} (59% of the baseline sodium current of 34 μA cm^{-2} in these 12 tissues).

Effects of A23187 on the isolated sodium current in sheep trachea

Mucosal addition of A23187 resulted in a dose-related reduction in I_{sc} with maximal effect 38 (6)% at 10 μM and IC_{50} 1 μM (Fig. 1*A*). No consistent change in g was observed although a significant reduction was seen at both 10 and 100 μM-A23187 (Fig. 1*B*). The time course of this effect at 10 μM is shown in Fig. 2. The response was significantly ($P < 0·05$) different from controls 7·5 min following addition of A23187. In paired experiments, pretreatment with 100 μM-amiloride ($n = 6$) significantly ($P < 0·05$) reduced the maximal inhibitory effect of 10 μM-A23187 from 7 (2) to 3 (1) μA cm^{-2} (Fig. 2) but the A23187 effect remained significantly ($P < 0·05$) different from baseline values from 10 min after its addition.

Effects of PDB on the isolated sodium current in sheep trachea

Mucosal addition of PDB caused a dose-related inhibition of I_{sc} (Fig. 3*A*). At concentrations up to 100 nM this was accompanied by a small but insignificant

decrease in g. At higher concentrations, further falls in I_{sc} were accompanied by large rises in g suggestive of tissue disruption (Fig. 3*B*). The time course of this PDB effect is shown in Fig. 4, the response becoming significantly ($P < 0.05$) different from controls 7·5 min following addition of PDB. On some occasions a small immediate rise in I_{sc} occurred on addition of PDB, but this was not statistically significant when

TABLE 1. Effects of pretreatment protocols used to isolate the sodium current in sheep tracheal ($n = 225$ tissues, 57 sheep) and human bronchial ($n = 22$ tissues, 4 individuals, between 2 and 7 tissues obtained from each lung) epithelium

	Sheep trachea	Human bronchus
Baseline I_{sc} (μA cm^{-2})	58 (2) (100%)	24 (2) (100%)
I_{sc} after acetazolamide (μA cm^{-2})	54 (2) (93%)	21 (2) (91%)
I_{sc} after bumetanide (μA cm^{-2})	46 (1) (79%)	18 (2) (77%)
I_{sc} after phloridzin (μA cm^{-2})	40 (1) (69%)	47 (2) (74%)

Results are expressed as mean (S.E.M.) of pooled results. The short circuit current (I_{sc}) expressed as a percentage of the starting value is shown below the absolute figure.

compared to controls. Mucosal addition of the stereoisomer 4α-PDB had no significant effect on I_{sc} at this concentration (Fig. 4). Serosal addition of 10 nM-PDB produced an inhibition in I_{sc} of 4 (2·2)% ($n = 5$) which was not significantly different from the ethanol-treated controls. For further studies a PDB concentration of 10 nM was chosen, as this was the highest concentration to produce a consistent fall in g, large rises in g occasionally occurring at 50 nM. In paired experiments, pretreatment with amiloride (100 μM) ($n = 6$) significantly ($P < 0.05$) reduced the inhibitory effect of 10 nM-PDB from 12·0 (4) to 1·2 (0·6) μA cm^{-2}, a response which was not significantly different from 4α-PDB-treated controls (Fig. 4).

Effects of the protein kinase C inhibitor calphostin C on the PDB-induced inhibition of the isolated sodium current and on C-kinase activity in sheep trachea

In preliminary experiments performed to attempt to block the PDB-induced inhibition of the sodium current using the putative PKC inhibitor calphostin C no consistent effect was observed. Therefore, the effect of calphostin C on PKC-catalysed phosphorylation of histone HIIIs in cell-free extracts of sheep tracheal epithelium was examined. In these studies the activity of PKC in the absence of calphostin was 87·2 pmol min^{-1} (mg protein)$^{-1}$. This was not significantly reduced (81·2 pmol min^{-1} (mg protein)$^{-1}$) in the presence of calphostin (10 μM).

Effects of forskolin and zardaverine on the isolated sodium current in sheep trachea

Serosal addition of forskolin (1 μM) and zardaverine (100 μM) ($n = 6$) produced an immediate small but significant ($P < 0.01$) rise in I_{sc} of 2·2 (0·02) μA cm^{-2} (6% of I_{sc}), followed by a gradual fall in I_{sc} of 1·4 (0·7) μA cm^{-2} (4% of I_{sc}). A small but significant rise in g of 0·25 (0·06) mS cm^{-2} occurred, which persisted 30 min after

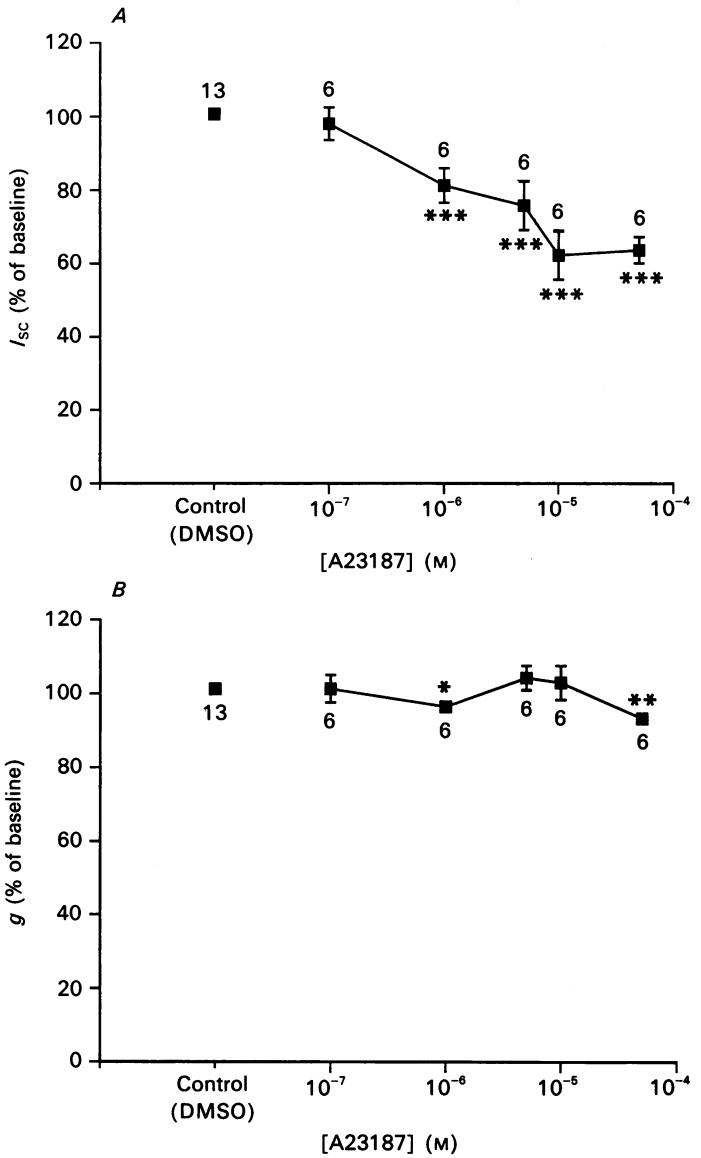

Fig. 1. Dose–response curves showing the effect of mucosal addition of A23187 on short circuit current, I_{sc} (*A*) and conductance, *g* (*B*) when applied to the isolated sodium current in sheep trachea. Error bars indicate S.E.M. and where not shown fall within the figure symbol. Tissue numbers are indicated at each point. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$ (intervention compared to DMSO-treated control).

addition of forskolin and zardaverine in spite of the fall in I_{sc}. In paired experiments, these responses remained significant ($P < 0{\cdot}05$) following pretreatment of tissues with amiloride (100 μM) ($n = 6$).

Effects of sequential addition of A23187, PDB and forskolin and zardaverine on the isolated sodium current in sheep trachea

Addition of A23187 (10 μM) 30 min after addition of PDB (10 nM) ($n = 6$) reduced the sodium current by a further 7 (2) μA cm^{-2} (18% of baseline I_{sc}) (Fig. 5), an effect

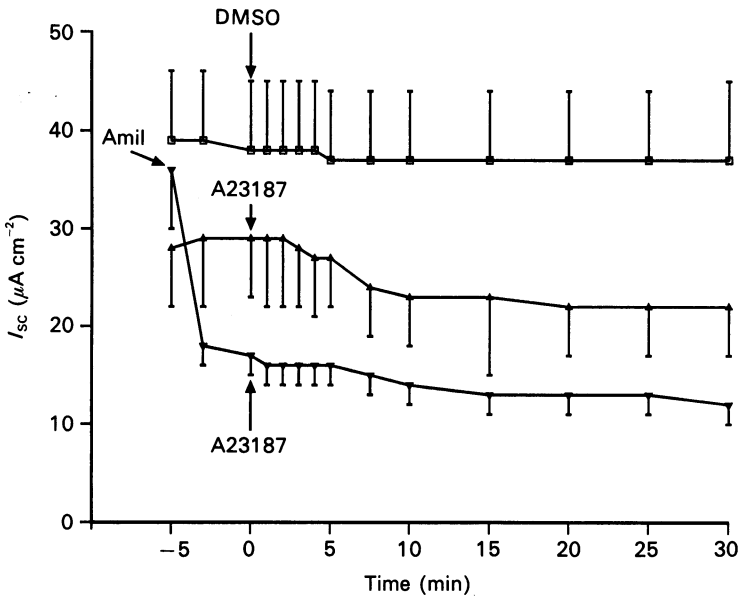

Fig. 2. Time course of the effect of mucosal addition of A23187 (10 μM) on the isolated sodium current, with and without amiloride pretreatment in sheep trachea ($n = 6$ for each intervention). Error bars indicate S.E.M. □, DMSO (diluent, 1 in 1000); ▲, A23187 (10 μM); ▼, A23187 (10 μM) following amiloride (Amil, 100 μM).

which was not significantly different from the reduction occurring with A23187 alone (7 (1) μA cm^{-2}, $n = 24$) in other unpaired experiments. Similarly, addition of PDB (10 nM) 30 min following addition of A23187 (10 μM) ($n = 6$) resulted in a fall in I_{sc} of 10 (2·4) μA cm^{-2} (36% of baseline I_{sc}) (Fig. 5), again not significantly different from the reduction occurring with PDB alone (12 μA cm^{-2} fall, $n = 24$) in other unpaired experiments. After pretreatment with both PDB (10 nM) and a combination of forskolin (1 μM) and zardaverine (100 μM) ($n = 6$) the effect of 10 μM-A23187 was a fall in I_{sc} of 5 (1) μA cm^{-2}, not significantly different from the 7 (1) μA cm^{-2} fall occurring in non-pretreated paired tissues (Fig. 5). Addition of PDB (10 nM) after both A23187 (10 μM) and forskolin (1 μM) with zardaverine (100 μM) ($n = 6$) resulted in a fall in I_{sc} of 14 (3) μA cm^{-2}, again not significantly different from the 14 (3) μA cm^{-2} fall occurring in paired tissues on addition of 10 nM-PDB alone.

Effects of forskolin and zardaverine, A23187 and PDB on intracellular cyclic AMP levels in sheep tracheal epithelium

Addition of forskolin (1 μM) and zardaverine (100 μM) to sheep trachea resulted in a mean 35-fold rise in intracellular cyclic AMP levels to 14·5 (5·4) mg (pg wet weight)$^{-1}$ ($n = 5$) compared to 0·4 (0·03) pg (mg wet weight)$^{-1}$ ($n = 5$) in diluent-treated controls. Intracellular cyclic AMP levels after A23187 ($n = 6$) and PDB

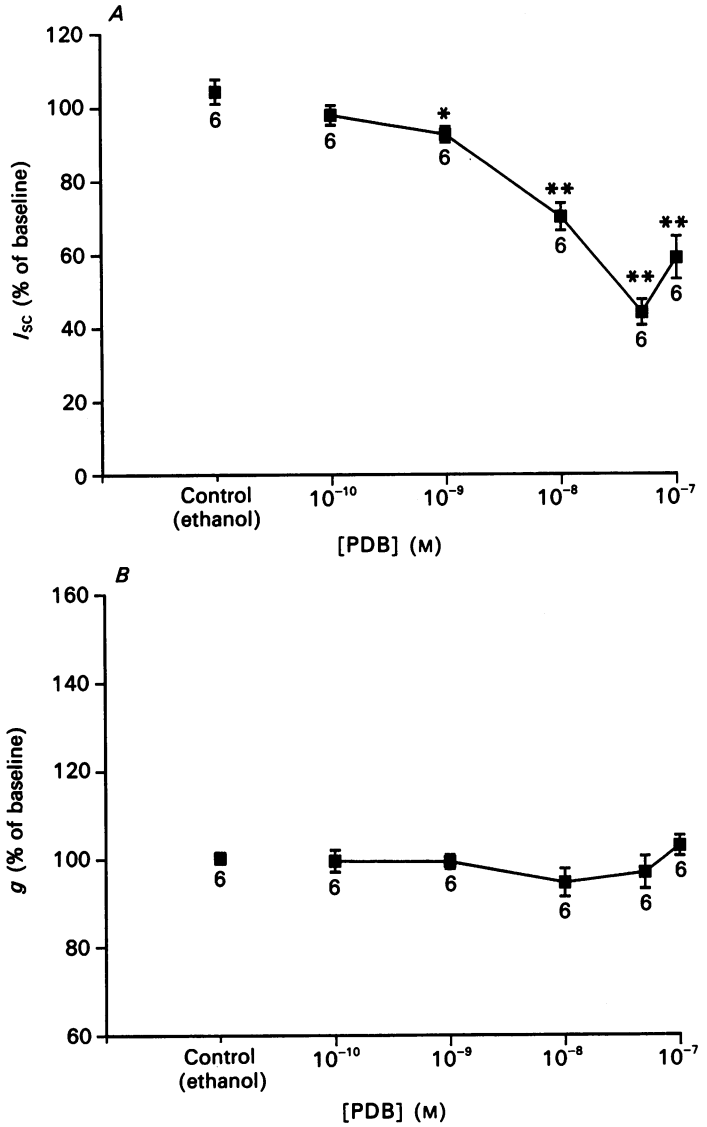

Fig. 3. Dose–response curves showing the effects of mucosal addition of 12,13-phorbol dibutyrate on short circuit current, I_{sc} (*A*) and conductance, g (*B*) when applied to the isolated sodium current in sheep trachea. Error bars indicate S.E.M. Tissue numbers are indicated at each point. $*P < 0.05$, $**P < 0.01$ (intervention compared to ethanol-treated control).

($n = 6$) were 0·5 (0·1) and 0·6 (0·1) pmol (mg wet weight)$^{-1}$ respectively, values not significantly different from those measured in diluent-treated controls (0·7 (0·2) for DMSO and 0·4 (0·1) for 4α-PDB).

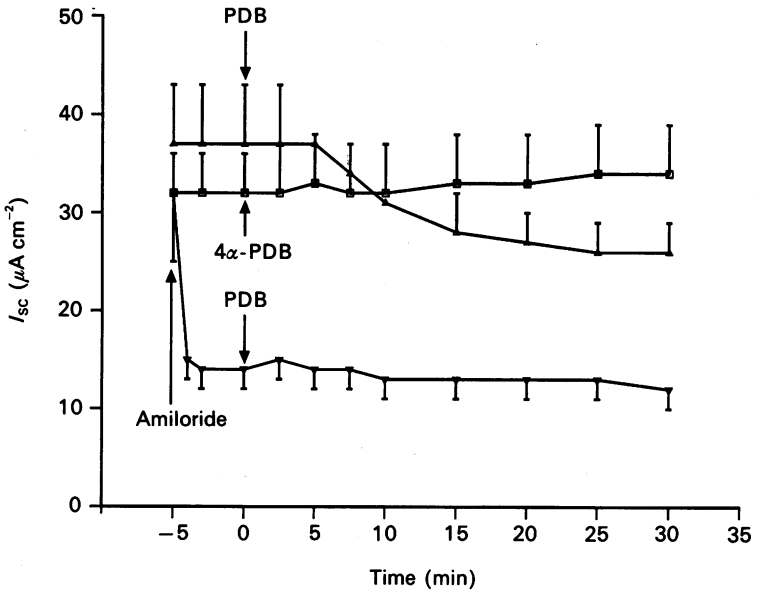

Fig. 4. Time course of effect of mucosal addition of 12,13-phorbol dibutyrate (PDB) with and without amiloride pretreatment when applied to the isolated sodium current in sheep trachea ($n = 6$ for each intervention). Error bars indicate S.E.M. □, 4α-PDB (10 nM); ▲, PDB (10 nM); ▼, PDB (10 nM) following amiloride (10 μM).

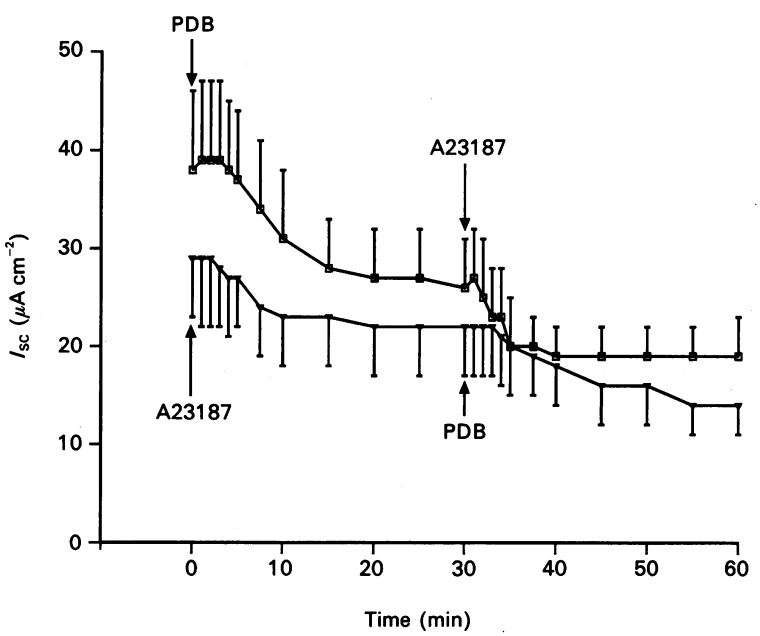

Fig. 5. Effects on the isolated sodium current of mucosal addition of A23187 (10 μM) following 12,13-phorbol dibutyrate (PDB) (10 nM) and of PDB (10 nM) following A23187 (10 μM) in sheep tracheal epithelium. □, PDB (10 nM) followed by A23187 (10 μM); ▼, A23187 (10 μM) followed by PDB (10 nM).

TABLE 2. Summary of the effects of A23187 (10 μM), 12,13-phorbol dibutyrate (PDB) (10 nM) and forskolin (1 μM)–zardaverine (100 μM) on the isolated sodium current in human bronchial epithelium ($n = 5$ tissues, 3 individual lungs, each intervention performed once or twice on tissue from one lung for all controls and interventions)

Intervention	Baseline I_{sc} (μA cm^{-2})	I_{sc} post-intervention (μA cm^{-2})	Reduction in I_{sc} (% of baseline sodium current)
Untreated control	14·6 (4·3)	13·6 (3·9)	
Sodium current control	20·7 (6·1)	18·6 (5·7)	10
10 μM-A23187	16·1 (3·9)	5·4 (1·8)**	66
10 nM-PDB	18·3 (1·6)	10·4 (0·7)**	43
1 μM-forskolin + 100 μM-zardaverine	16·4 (2·9)	15·7 (3·2)$^{n.s.}$	4

Results are expressed as mean (S.E.M.) of pooled results. **$P < 0·01$, n.s. = not significant (comparison of the change in I_{sc} following an intervention compared with that of adding diluent to the sodium current control).

TABLE 3. Effects of sequential addition of A23187 (10 μM), 12,13-phorbol dibutyrate (10 nM) and forskolin (1 μM) + zardaverine (100 μM) on the isolated sodium current in human bronchus ($n = 5$ tissues, 3 individual lungs, each intervention performed once or twice on tissue from one lung unless otherwise indicated)

Intervention	I_{sc} pre-intervention (μA cm^{-2})	I_{sc} post-intervention (μA cm^{-2})	Reduction in I_{sc} (% of baseline sodium current)
Untreated control	13·6 (3·9)	13·2 (3·9)	—
Sodium current control	18·6 (5·7)	16·1 (5·7)	12
10 μM-A23187 after 10 nM-PDB	10·4 (0·7)	3·2 (0·4)	39
10 μM-A23187 after 1 μM-forskolin + 100 μM-zardaverine ($n = 2$)	15·0	10·7	30
10 nM-PDB after 10 μM-A23187	5·4 (1·8)	2·9 (1·1)	18
10 nM-PDB after 1 μM-forskolin + 100 μM-zardaverine ($n = 3$)	15·7 (3·2)	8·2 (3·2)	50
1 μM-forskolin + 100 μM-zardaverine after 10 nM-PDB and 10 μM A23187	3·2 (0·4)	2·1 (0·8)	6
1 μM-forskolin + 100 μM-zardaverine after 10 μM-A23187 and 10 nM-PDB	2·9 (1·1)	2·1 (0·8)	4

Results are expressed as mean (S.E.M.) of pooled results. Comparison of the change in short circuit current (I_{sc}) occurring with sequential addition of a drug or drugs against that occurring with the intervention alone showed no differences, except in the case of addition of PDB after A23187 when the change in I_{sc} was significantly ($P < 0·01$) reduced.

Effects of A23187, PDB and forskolin and zardaverine on the isolated sodium current in human bronchi

The effects of A23187 (10 μM), PDB (10 nM) and forskolin (1 μM) + zardaverine (100 μM) on human bronchi are summarized in Table 2, and compared with both untreated and isolated sodium current controls. The 43% inhibition of I_{sc} by PDB (10 nM) was not significantly different from its effect in sheep trachea (30% inhibition). A23187 (10 μM) produced a significantly ($P < 0·05$) greater inhibition of the sodium current in human bronchus (66%) than in sheep trachea (36%). In a

limited number of experiments using A23187 (5 μM) ($n = 2$) the sodium current was inhibited by 50 and 55% with no change in the single control. No stimulation of the sodium current was seen with forskolin and zardaverine and the 4% fall in I_{sc} was not significantly different from the 10% fall occurring in the sodium current controls.

The effects of A23187 (10 μM) and PDB (10 nM) in tissues which had already been treated with one of these agents or with forskolin (1 μM) and zardaverine (100 μM) are summarized in Table 3. With the exception of the effect of 10 nM-PDB following 10 μM-A23187, which was significantly ($P < 0.01$) reduced, the responses observed were not significantly different from those occurring on initial addition of these agents. Addition of forskolin (1 μM) and zardaverine (100 μM) to tissues that had been pretreated with PDB (10 nM) followed by A23187 (10 μM) and with A23187 (10 μM) followed by PDB (10 nM) again produced reductions in I_{sc} not significantly different from diluent-treated sodium current controls.

DISCUSSION

Interpretation of our study is dependent on two factors: firstly that our inhibitor protocol was predominantly acting on the ion transport processes targeted; and secondly that the pharmacological interventions were principally mediated through the individual second-messenger pathways.

With respect to inhibitor specificity, our previous studies (Steel *et al.* 1991*a*; Steel, Graham, Alton & Geddes, 1991*b*) have shown that phloridzin produces a dose-related reduction in I_{sc} only when applied to the mucosal surface. Substitution of glucose by mannitol results in a fall in I_{sc} not significantly different from that produced by phloridzin, the whole effect of which is abolished by this glucose substitution. It is unlikely that phloridzin is acting on intracellular processes such as ATP synthesis in this tissue because of the lack of effect of serosal addition in comparison to mucosal addition. These features are consistent with an inhibitory effect of phloridzin on sodium–glucose co-transport across the apical membrane.

At concentrations up to 100 μM, acetazolamide has been shown to be highly specific for the carbonic anhydrase-catalysed reaction (Maren, 1977) and was effective when applied either mucosally or serosally in our studies. Consequently, the fall in I_{sc} observed with acetazolamide relates either to a reduction in HCO_3^- secretion (for which an appropriately directed driving force of approximately 12 mV exists) or to a secondary effect of alteration in intracellular pH on other ion transport processes. Supportive of the former are previous microelectrode studies using sheep trachea, showing a very similar fall in I_{sc} induced by bathing tissues in HCO_3^- and CO_2-free solutions (Acevedo, Olver & Ward, 1990). Pretreatment with acetazolamide did not alter the magnitude of the subsequent amiloride or sodium substitution response, nor that of bumetanide or phloridzin. Similarly, the acetazolamide effect was not altered by pretreatment with bumetanide or phloridzin. This suggests that the acetazolamide effect is not secondary to alterations in other ion transport processes.

Bumetanide is a potent inhibitor of the Na^+–K^+–$2Cl^-$ co-transporter in several tissues with an IC_{50} closely comparable to that found in our study (O'Grady, Palfrey & Field, 1987). Bumetanide may also block apically located chloride channels

(Landry, Reitman, Cragoe & Al-Awqati, 1987), an effect which would serve to increase its desired efficacy in these studies.

Although amiloride is known to inhibit many ion transport processes (Na^+–K^+-ATPase, Na^+–Ca^{2+} exchange and Na^+–glucose co-transport), the IC_{50} values for these are in the millimolar range (Kleyman & Cragoe, 1988). However, the IC_{50} for the effect of amiloride on the Na^+–H^+ antiport is in the range 3–84 μM in a variety of tissues. We therefore studied the effect of a specific inhibitor of this antiport 5-[*n*-ethyl-*n*-isopropyl]-amiloride (EIPA) on sheep trachea. The time course (25 min) and magnitude (25% inhibition of I_{sc}) of effect of EIPA are markedly different from that of amiloride.

In earlier studies we have demonstrated that sodium substitution with choline does not alter the subsequent effect of bumetanide or phloridzin, although producing a small reduction in the effect of acetazolamide. This manoeuvre does not therefore have a major secondary effect on any of the known ion transport processes in this tissue. The portion of the I_{sc} removed by sodium substitution with choline following pretreatment with acetazolamide, bumetanide and phloridzin may be attributed to sodium transport through amiloride-insensitive sodium channels. These have recently been demonstrated in airway epithelia (Duszyk & French, 1989; Chinet, Fullton, Boucher & Stutts, 1991). However, the possibility that some of the amiloride-insensitive sodium current may represent presently unidentified sodium-dependent ion transport processes cannot be excluded. Thus, although no presently available inhibitors are entirely specific, it is likely that the 'isolated sodium current' did not contain significant contributions from other known ion transport processes.

A23187 has been used as a calcium ionophore in a large number of studies of epithelial ion transport, although measurement of intracellular calcium has been hindered by the fluorescent nature of this compound. However, ionomycin produces very similar effects (Willumsen & Boucher, 1989) and has been shown to directly elevate intracellular calcium in human nasal epithelium (Murphy, Cheng, Yankaskas, Stutts & Boucher, 1988). No stimulation of cyclic AMP levels by A23187 was seen in our tissues. Furthermore, the effects of A23187 were not influenced by previous application of the other two agonist protocols.

Phorbol esters have been shown to activate PKC directly (Castagna, Takai, Kaibushi, Sano, Kikkawa & Nishizuka, 1982), although other effects such as activation of phospholipase D in lymphocytes have been reported (Yu-Zhang, Reddy & Mastro, 1990). The lack of effect of the PDB stereoisomer 4α-PDB lends support to PKC as the substrate for the PDB effect since the activation of PKC by its physiological activator diaylglycerol (DAG) is also stereospecific, only 1,2-*sn* stereoisomers of DAG being active (Nishizuka, 1986). Also, PDB had no effect on intracellular cyclic AMP levels and its effect on the sodium current was not influenced by previous treatment of the tissues with A23187 or forskolin and zardaverine. The lack of effect of the putative PKC inhibitor calphostin C in cell-free extracts suggests that this inhibitor is not suitable for studies in sheep tracheal epithelium. This may relate to lack of selectivity for the PKC isoforms present in airway epithelium.

The combination of forskolin and zardaverine produced a 35-fold increase in intracellular cyclic AMP levels in sheep tracheal epithelium and this rise has been

shown to be associated with chloride secretion in other studies (Steel, Graham, Alton & Geddes, 1991*c*). The bioelectric response to forskolin and zardaverine was qualitatively and quantitatively different from those seen in either of the above stimulation protocols and in the presence of forskolin and zardaverine both A23187 and PDB exerted their full effect.

Thus, in this study we have shown that stimulation of two second-messenger pathways, namely elevation of intracellular calcium with A23187 and stimulation of PKC with PDB have marked inhibitory effects on sodium transport in both sheep trachea and human bronchus. The effect of stimulation of the A kinase pathway on sodium transport in sheep trachea is small and is not significant in human bronchus in spite of large increases in intracellular cyclic AMP levels.

Elevating intracellular calcium with calcium ionophores is known to inhibit sodium transport in a number of epithelia including toad bladder (Ludens, 1978) and rat and rabbit cortical collecting tubule (Palmer & Frindt, 1987; Frindt & Windhager, 1990). A23187 has also been shown to inhibit sodium transport in canine tracheal epithelium (Cullen & Welsh, 1987). Our results provide new evidence for an inhibitory effect of elevation of intracellular calcium on sodium transport in sodium-absorbing conducting airways. Elevating intracellular calcium is also known to stimulate basolateral potassium conductance in airway epithelium. However, such changes would if anything result in alterations in the intracellular driving forces favouring, rather than inhibiting Na^+ absorption.

Phorbol ester-induced stimulation of PKC produces variable effects on sodium transport in a number of tissues including stimulation of amiloride-sensitive sodium transport in frog skin (Civan, Rubenstein, Mauro & O'Brien, 1985; Civan, Peterson-Yantorno & O'Brien, 1987) and inhibition of sodium transport in other tissues including frog urinary bladder (Satoh & Endou, 1990), A6 renal cells (Yanase & Handler, 1986) and rabbit cortical collecting tubule (Ando, Jacobson & Breyer, 1987). Our results with PDB at a similar concentration (20 ng ml^{-1} or 40 nM) to that applied to A6 cells also showed an inhibitory effect, which we believe is the first report of inhibition of airway epithelial sodium transport by phorbol ester-induced PKC stimulation.

The A-kinase pathway has been shown to stimulate sodium absorption in several sodium transporting epithelia including frog skin, toad bladder and rat kidney cortical collecting tubules (Garty & Benos, 1988). In pulmonary tissue this pathway stimulates sodium absorption by the whole lung in the fetal lamb (Olver, Ramsden, Strang & Walters, 1986; Olver, Ramsden & Walters, 1987) and the adult rat (Goodman, Anderson & Clemens, 1989). In canine trachea, studies investigating the regulation of sodium absorption using bilateral chloride-free bathing solutions suggest this pathway may stimulate sodium absorption in conducting airways (Cullen & Welsh, 1987). Our studies do not distinguish between the possibilities that conducting airways are under maximal tonic stimulation by this pathway or that such airways in a sodium absorbing tissue are relatively unresponsive to this second-messenger pathway.

Our studies provide no information on the mechanisms linking these intracellular pathways with changes in apical membrane sodium permeability in airway epithelia. Local regulatory pathways may be important, as in A6 cells, where pertussis toxin-

sensitive G proteins have a role in regulating sodium transport across the apical membrane (Cantiello, Patenaude & Ausiello, 1989). In both A6 cells and intact bovine renal collecting tubules immunochemical studies have localized the amiloride-sensitive sodium channel only to the apical membrane, where it is present in activated and inactivated forms (Tousson, Alley, Sorscher, Brinkley & Benos, 1989). The local factors regulating the level of activation of these channels and their links with intracellular regulatory pathways remain to be established. The demonstration of inhibition of sodium transport by two major intracellular regulatory pathways in this study provides information on further potential sites for pharmacological intervention in disorders of airway ion transport.

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