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Differential regulation of Na^+ and Cl^- conductances by PTX-sensitive G proteins in fetal lung apical membrane vesicles

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Abstract

In apical membrane vesicles (AMV) prepared from late gestation fetal guinea pig lung we show that conductive $^{22}Na^+$ uptake is modulated by at least two pathways involving pertussis toxin (PTX)-sensitive G proteins. Intravesicular incorporation of 100 μM GTPγS into vesicles resuspended in NaCl caused a significant stimulation ($P < 0.05$) of conductive Na^+ uptake in AMV to 150 ± 10% ($n = 10$) of control, whereas GDPβS reduced uptake to 65 ± 9% ($n = 4$) of control. This contrasting response to GTPγS and GDPβS is characteristic of a G protein mediated pathway. GTPγS induced a significantly smaller stimulation, 125 ± 8% ($n = 5$) of control, in the presence of the relatively impermeant anion isethionate (Ise^-). Taken together, these data indicate modulation of both Na^+ and Cl^- channels in the apical membrane by co-localised G protein(s). Treatment with PTX stimulated conductive $^{22}Na^+$ uptake to 171 ± 20% ($n = 13$) of control in AMV resuspended in NaCl, but did not have a significant effect, 94 ± 19% of control, in the presence of NaIse indicating the existence of tonic activation of Cl^- channels in these AMV under resting conditions. As the combined effects of PTX and GTPγS diminished uptake, we propose that the G protein(s) responsible for Na^+ channel activation in response to GTPγS is PTX-sensitive and that additional PTX-insensitive G proteins might also modulate $^{22}Na^+$ uptake in these AMV. The presence of $G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$ and $G_o\alpha$ in this apical membrane preparation was confirmed by PTX catalysed [^{32}P]ADP-dependent ribosylation and Western blotting. Incubation of AMV with 200 μM DTT caused an inhibition of conductive Na^+ uptake in AMV resuspended in NaCl or NaIse to 66 ± 8% ($n = 11$) and 64 ± 8% ($n = 6$) of control respectively. Pre-treatment with DTT did not affect the ability of GTPγS to stimulate conductive Na^+ uptake suggesting that the regulation of $^{22}Na^+$ uptake in late gestation guinea pig fetal lung AMV is unlikely to involve an associated regulatory protein.

Keywords: Alveolar epithelium; Sodium transport; Sodium channel; Chloride channel; G protein

1. Introduction

Modulation of ion and water transport across the pulmonary epithelium during development is essential for lung morphogenesis and function. Around the time of birth the ion transport properties of the epithelium switch from being predominantly Cl^- se-

Abbreviations: NAD, nicotinamide adenine dinucleotide phosphate; TRA, triethanolamine; Tris, tris(hydroxymethyl)aminomethane

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creting to Na^+ absorbing with transepithelial Na^+ movement from the lung lumen driving fluid reabsorption as the lung takes on the function of gas exchange [1]. Fluid reabsorption in the fetus [1] is cAMP-dependent [2] and blockable by amiloride and its structural analogues. This in-vivo evidence, together with the demonstration of cAMP stimulated amiloride-sensitive Na^+ transport [3–5] in alveolar type II (ATII) cells and the immunological [6] and functional [7] localisation of amiloride-sensitive sodium channels to the apical membrane of ATII cells, implicates this cell type in the process of Na^+ driven fluid reabsorption in the lung.

Fetal catecholamines elevated by the stress of labour have been shown to play a role in the regulation of amiloride-sensitive Na^+ transport at birth [1,8]. β-Adrenoceptor activation stimulates both lung fluid absorption and net sodium flux from the lung lumen to the interstitium [1]. There is evidence to suggest that this adrenaline induced stimulation of Na^+ uptake is transduced by the stimulatory G protein, Gs associated with the receptor on the basolateral membrane of the ATII cell [9,10] and involves the adenylate cyclase/cAMP pathway [2]. This is unlike the regulation of Na^+ transport through amiloride-sensitive epithelial sodium channels in other water absorbing epithelia, such as the kidney [11–13], in which apically located pathways involving PTX-sensitive G proteins have been implicated.

We have previously identified a Na^+-selective channel ($P_{Na+}/P_{K+} = 1.8 \pm 0.1$) with relatively low sensitivity to amiloride, in excised membrane patches from ATII cells freshly isolated from late gestation fetal guinea pig lung. This channel can be shown to be modulated by activation and inhibition of G proteins with the non-metabolisable analogues of GTP and GDP (GTPγS and GDPβS) respectively [14]. Evidence of G protein modulation of $^{22}Na^+$ uptake in apical membrane vesicles (AMV) prepared from late gestation fetal guinea pig lungs [7] indicates that the Na^+ channel and associated G protein(s) reside in the ATII cell apical membrane.

Here, we provide immunological evidence for the presence of a number of PTX-sensitive and -insensitive G protein isoforms in the fetal ATII cell apical membrane and, using a modification of the protocol we have previously described [7] for quantifying $^{22}Na^+$ uptake into AMV, we report the result of studies undertaken to examine how G proteins regulate conductive $^{22}Na^+$ uptake by modulation of co-localised Na^+ and Cl^- conductance pathways.

Some of this work has been presented previously in abstract form.

2. Materials and methods

2.1. Apical membrane preparation

Lung apical membrane was prepared from 1–3 day preterm (F1–3) guinea pigs (term = 68 days) as described previously [7]. Apical membrane was resuspended in NaCl buffer (125 mM NaCl, 10 mM TRA, pH 7.6) or sodium isethionic acid (NaIse) buffer (125 mM $C_2H_5O_4SNa$, 10 mM TRA, pH 7.6) and stored at −70°C. Protein content was estimated using the Bradford method, with bovine serum albumin as a standard. Alkaline phosphatase activity (EC 3.1.3.1), a marker for brush border membrane, showed a mean enrichment of 16.5 ± 2.5, $n = 15$. Specific markers of mitochondrial (succinate-dependent cytochrome *c* reductase activity, EC 1.3.99.1), and endoplasmic reticular membranes (NADPH-dependent cytochrome *c* reductase activity, EC 1.6.2.4) showed no enrichment, whilst the lysosomal membrane marker (acid phosphatase activity, EC 3.1.3.2) enrichment was small but consistently more than unity conforming with previously published results [7].

2.2. $^{22}Na^+$ uptake assays

The uptake assay was adapted by this laboratory [7] from a protocol by Garty et al. [15]. Briefly, this protocol amplifies conductive transport via cation-specific channels. The removal and replacement of external Na^+ with the impermeable cation $Tris^+$ results in an outwardly directed Na^+ gradient and the establishment of an electrical diffusion potential resulting from the increased permeability of Na^+ relative to that of Cl^- or $Tris^+$, which in turn drives tracer accumulation. The difference between $^{22}Na^+$ uptake with and without an imposed outward gradient represents conductive Na^+ uptake (i.e. it is assumed that electroneutral transport or Na^+/solute co-transport [7] is eliminated). The initial rate of $^{22}Na^+$ uptake is estimated at 2 min, at which point

extravesicular $^{22}Na^+$ is stripped off the vesicles using Dowex 50W-X8 (50–100 μm mesh, Tris form) ion exchange columns. All data are expressed in terms of initial rate conductive $^{22}Na^+$ uptake.

In the presence of outwardly directed ion gradients, the Goldman-Hodgkin-Katz equation predicts that the magnitude of the intravesicular potential is determined by the relative permeability of the apical membrane to cations and anions. Thus, accumulation of $^{22}Na^+$ into the vesicles can be modulated by altering the permeability to Cl^- (changing the driving force) as well as to Na^+. The consequence of changing Cl^- permeability on conductive $^{22}Na^+$ uptake was examined by substituting Cl^- with isethionate$^-$ (Ise^-, $C_2H_5SO_4^-$), a larger, less permeant anion, and by the use of the Cl^- channel blocker 4-acetamido-4′-isothiocyaostilbene-2,2′-disulphonic acids (SITS). Conductive $^{22}Na^+$ uptake experiments in the presence of NaIse were performed as previously described, substituting NaIse for NaCl. SITS, 100 μM, was added to AMV resuspended in NaCl immediately after imposition of the Na^+ gradient. The results (Fig. 1) indicated that there is a significant Cl^- conductance in these AMV and, therefore, in order to differentiate between changes in $^{22}Na^+$ uptake due to changes in the electrical driving force via the anion conductance and those due to modulation of the Na^+ conductance, experiments were conducted using both NaCl and NaIse to generate outwardly directed Na^+ gradients.

The G protein modulators, 100 μM GTPγS or 100 μM GDPβS, were introduced at the vesiculation stage in the presence of 1 mM $MgCl_2$ and were left to equilibrate at room temperature for 1 h prior to imposition of the Na^+ gradient.

The data are presented as mean ± S.E.M. Statistical analysis was performed using the Student's *t*-test. $P \leq 0.05$ was considered significant.

2.3. DTT pre-treatment of apical membrane

Membrane vesicles were prepared by 20 passes through a 21 gauge needle in ribosylation buffer (105 mM NaCl, 10 mM TRA, 20 mM thymidine, 1 mM ATP, 20 mM arginine, 100 μM GTP, 3 μM NAD, pH 7.0) supplemented with 200 μM DTT. After a 1 h incubation at 37°C the AMV were diluted with 125 mM NaCl or NaIse and centrifuged at 70 000 × *g* for 45 min at 20°C. The final concentration of DTT in the AMV pellet was calculated to be 1.3 μM.

2.4. PTX-dependent ADP-ribosylation of apical membrane

ADP-ribosylation of apical membrane was carried out according to the protocol of McKenzie et al. [17]. PTX was pre-activated in activation buffer (115 mM NaCl, 10 mM TRA, 20 mM DTT, pH 8.0) for 1 h at 37°C [18]. 800 μg of apical membrane was vesiculated in 1 ml of ribosylation buffer with 10 μg of pre-activated PTX. ADP-ribosylation of membrane proteins, using NAD as a substrate, was carried out for 1 h at 37°C. After the incubation, the AMV were diluted with 125 mM NaCl and centrifuged at 70 000 × *g* for 45 min at 20°C. This procedure gave a final calculated concentration of 100 ng ml^{-1} PTX, 1.3 μM DTT inside the AMV. The proteins ribosylated by PTX were investigated and optimal incubation time was assessed as giving maximum ribosylation using the same protocol with [^{32}P]NAD as the substrate. Labelled protein samples of 30 μg were resolved by SDS-PAGE using 10% acrylamide 0.26% w/v resolving gels. Gels were dried and exposed to autoradiographic film for up to 1 week. Quantitation of labelling was assessed by densitometry scanning of blots.

2.5. Western blotting of G proteins

Apical membrane proteins (40 μg) were separated by SDS-PAGE. Resolving gels and running conditions varied according to which G protein α-subunit was being investigated. $G_i\alpha_3$: 10% acrylamide, 0.26% bis w/v run at 150 V for 2 h; $G_i\alpha_{1/2}$ and $G_{q/11}\alpha$ isotypes: 12.5% acrylamide, 0.065% bis supplemented with 6 M urea run at 5 mA for 17 h; $G_o\alpha$ isotypes: 9% acrylamide, 0.26% bis with 6 M urea run at 5 mA for 17 h. Detection of the G protein α-subunits was performed by Western blotting with specific antipeptide antisera. The proteins were transferred to nitrocellulose (Schleicher and Schuell, Germany) by semi-dry electrophoresis at 0.8 mA/cm^2 using a discontinuous buffer system. Cathode buffer: 25 mM Tris-base, 129 mM glycine, 20% v/v methanol, 0.2% w/v SDS. Anode buffer: 25 mM Tris-base,

129 mM glycine, 25% v/v methanol. The filters were blocked using 5% w/v non-fat dry milk in PBS+0.05% sodium azide (PBS/azide) for 1 h at room temperature prior to incubation with primary antiserum, diluted 1:500 in 1% bovine serum albumin in PBS/azide, overnight at 4°C. After removal of primary antiserum, visualisation of the immunologically detected α-subunits was achieved by the use of a horseradish peroxidase coupled goat anti-rabbit IgG secondary antibody (Sigma, Poole, UK), followed by development with ECL-Western blotting detection system (Amersham Int., Amersham, UK) using standard protocols. ECL-stained blots were exposed to X-ray film overnight. The antisera used in these experiments were SG1, raised to a carboxy-terminal decapeptide common to $G_i\alpha_1$ and $G_i\alpha_2$ but not $G_i\alpha_3$, ON1 raised against the N-terminal decapeptide common to $G_o\alpha_1$ and $G_o\alpha_2$ [19] kindly supplied by G. Milligan (Glasgow). Antibodies specific to $G_i\alpha_3$ and $G_q\alpha/G_{11}\alpha$ were purchased from Calbiochem (Nottingham, UK).

2.6. Reagents

All reagents were of the highest grade available and purchased from Sigma (Poole, Dorset, UK) unless stated otherwise. Protein dye reagent was purchased from Bio-Rad Laboratories (Hemel Hempstead, Hertfordshire, UK). ^{22}NaCl (carrier free; specific activity 1004 mCi mg^{-1}) was purchased from NEN-Du Pont (Stevenage, Hertfordshire, UK). Disposable Pasteur pipettes were purchased from Alpha Labs (Eastleigh, Hampshire, UK).

3. Results

3.1. Conductive $^{22}Na^+$ uptake in apical membrane vesicles is modulated by anion permeability

The initial rate of conductive $^{22}Na^+$ uptake in AMV resuspended in NaCl was 34.6 ± 5.5 pmol (mg protein)$^{-1}$ (n = 8) and 56.0 ± 7.2 (n = 8) in AMV resuspended in Ise$^-$. These values were considered a measurement of the basal conductive $^{22}Na^+$ uptake and represent 100% control levels to which the experimental manipulations are compared. Substitution of NaCl with NaIse thus increased uptake 180 ± 8% (n = 8). Addition of 100 μM SITS after gradient imposition with NaCl increased conductive $^{22}Na^+$ uptake to a level similar to that observed with NaIse: 173 ± 7% of control (n = 6) (Fig. 1). These data indicate that the AMV possess a significant anion conductance and thus, for the reasons discussed in Section 2, subsequent experiments included a comparison of $^{22}Na^+$ uptake between vesicles in which the gradient was imposed by NaCl and those in which NaIse was used.

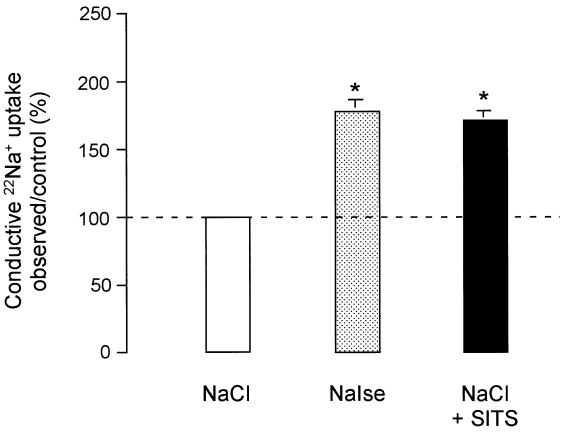

Fig. 1. Conductive $^{22}Na^+$ uptake in AMV is modulated by anion permeability. Apical membranes were revesiculated in the presence of 125 mM NaCl, 125 mM NaIse or 125 mM NaCl in the presence of 100 μM SITS. Conductive $^{22}Na^+$ uptake was measured at 2 min and is expressed as a percentage relative to NaCl control (100%). Replacing NaCl with NaIse (n = 8) or the addition of SITS to the extravesicular solution (n = 6) increased conductive $^{22}Na^+$ uptake. *Significantly different from control, $P < 0.05$.

3.2. GTPγS stimulates conductive $^{22}Na^+$ uptake in AMV containing NaCl or NaIse

The intravesicular incorporation of 100 μM GTPγS in AMV resuspended in NaCl caused a significant stimulation of conductive $^{22}Na^+$ uptake to 150 ± 10% of control (100%) (n = 10, $P < 0.05$) whereas 100 μM GDPβS caused an inhibition of conductive $^{22}Na^+$ uptake to 65 ± 9% of control (n = 4, $P < 0.05$) (Fig. 2). This contrasting response to the GTP analogues is characteristic of G protein mediated pathways in accordance with our previous findings [16]. When the effect of GTPγS was studied in AMV resuspended in NaIse, we again observed a stimulation of conductive $^{22}Na^+$ uptake but the

response was smaller (125 ± 5% of control; $n = 5$, $P = 0.05$) than that seen in the presence of NaCl, suggesting that intravesicular incorporation of GTPγS does indeed increase conductive $^{22}Na^+$ uptake through inhibition of Cl^- selective pathways (increasing the electrical driving force) in addition to activating Na^+ channels. These data are consistent with our previous findings in excised patches of ATII cells of similar gestational age [14,27].

3.3. DTT has similar effects on conductive $^{22}Na^+$uptake in the presence of NaCl and NaIse

The thiol reducing agent DTT is required for the activation of PTX and has previously been shown to dissociate regulatory pathways associated with the amiloride-sensitive epithelial sodium channel [20,21]. Pre-treatment of the apical membrane with 200 μM DTT caused a significant inhibition of conductive $^{22}Na^+$ uptake to 66 ± 8% of control in NaCl AMV ($n = 11$, $P < 0.05$) (Fig. 3). DTT caused a similar inhibition of conductive $^{22}Na^+$ uptake to 64 ± 13% ($n = 6$, $P < 0.05$) of control in AMV resuspended in NaIse suggesting that the effect of DTT is specific to Na^+ selective pathways and independent of Cl^- permeability.

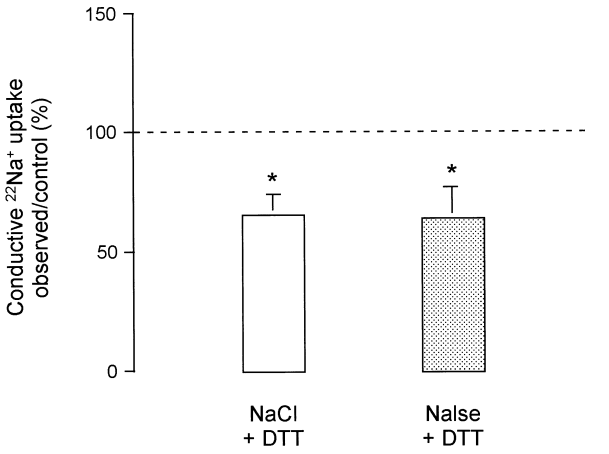

Fig. 3. DTT inhibits conductive $^{22}Na^+$ uptake in the presence of NaCl or NaIse. Apical membranes were vesiculated in the presence or absence of 200 μM DTT and incubated at 37°C for 1 h. The effect of DTT in the presence of 125 mM NaCl ($n = 11$) or 125 mM NaIse ($n = 6$) is expressed as a percentage relative to control conductive uptake levels for each anion, NaCl or NaIse (100%). *Significantly different from control, $P < 0.05$.

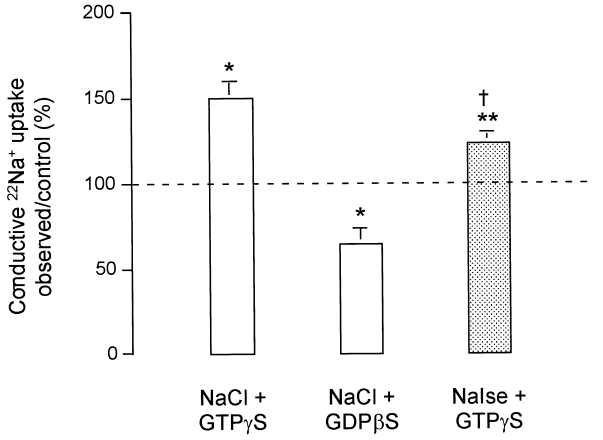

Fig. 2. Activation of G proteins stimulates conductive $^{22}Na^+$ in AMV containing either NaCl or NaIse. Apical membranes were revesiculated in 125 mM NaCl in the presence of either 100 μM GTPγS, an irreversible G protein activator ($n = 10$), or GDPβS, an irreversible inhibitor of G proteins ($n = 4$). Apical membranes were also revesiculated in 125 mM NaIse in the presence of GTPγS ($n = 6$). The effects of GTPγS and GDPβS are expressed as a percentage of control conductive uptake levels for the anion used, i.e. NaCl or NaIse. *Significantly different from NaCl control, $P < 0.05$. **Significantly different from NaIse control, $P = 0.05$. †Significantly different to the effect of GTPγS in the presence of 125 mM NaCl, $P < 0.05$.

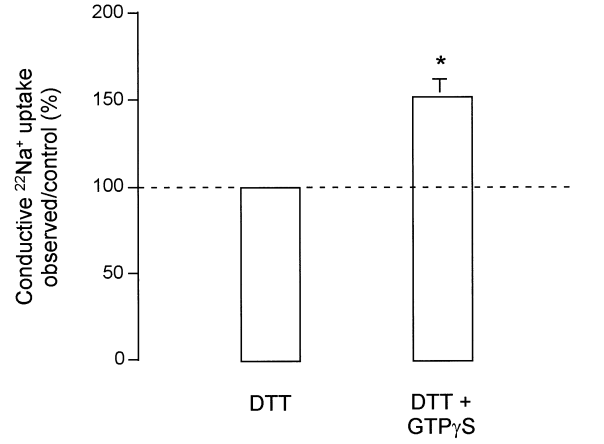

Fig. 4. GTPγS stimulates conductive $^{22}Na^+$ uptake in the presence of DTT. Apical membranes were vesiculated in 125 mM NaCl, in the presence or absence of 200 μM DTT and incubated at 37°C for 1 h, then revesiculated in the presence or absence of 100 μm GTPγS and allowed to equilibrate for 1 h at room temperature. The effect of GTPγS in the presence of DTT ($n = 15$) is expressed as a percentage relative to control conductive uptake levels in the presence of DTT (100%). *Significantly different from control, $P < 0.05$.

3.4. DTT does not affect GTPγS stimulation of conductive $^{22}Na^{+}$uptake

To determine whether DTT had any effect on the GTPγS modulated pathway in AMV, experiments similar to those already described for GTPγS were performed with apical membranes pre-treated with DTT. As both GTPγS and DTT had similar effects in AMV resuspended in NaCl or NaIse, these experiments were carried out in the presence of NaCl only. The stimulation of conductive $^{22}Na^{+}$ uptake achieved by GTPγS in the presence of DTT was $152 \pm 10\%$ ($n = 15$), a level similar to that observed in untreated AMV (Fig. 4). Thus, DTT did not affect the ability of GTPγS to stimulate conductive $^{22}Na^{+}$ uptake.

3.5. The effect of PTX-dependent ADP-ribosylation on conductive $^{22}Na^{+}$ uptake is mediated via changes in anion conductance

The use of PTX in cytosol-free preparations requires pre-activation of PTX by incubation with DTT. Therefore, experiments using PTX pre-treatment were controlled by comparison to pre-treatment with 200 μM DTT in a parallel limb. In AMV resuspended in NaCl, 10 μg ml^{-1} pre-activated PTX was predominantly stimulatory inducing an increase in conductive $^{22}Na^{+}$ uptake to $171 \pm 20\%$ of control levels ($n = 13$; $P < 0.05$) (Fig. 5). However, in AMV resuspended in NaIse, pre-treatment with PTX did not significantly affect conductive uptake, $94 \pm 19\%$ of control ($n = 7$). These data suggest that inhibition of PTX-sensitive G proteins by ADP-ribosylation most likely have a predominant effect on Cl^{-} permeability, inhibiting Cl^{-} conductance and thus increasing the driving force for conductive $^{22}Na^{+}$ uptake.

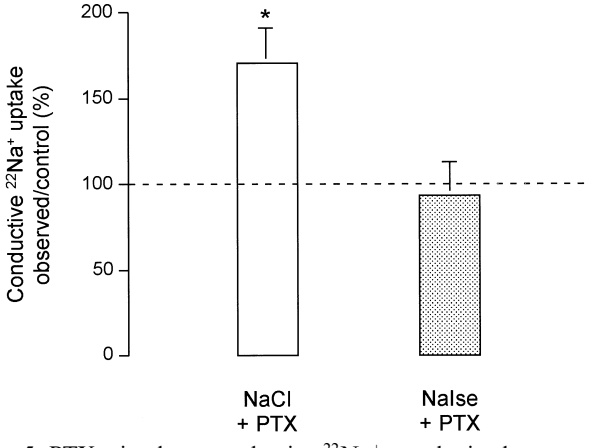

Fig. 5. PTX stimulates conductive $^{22}Na^{+}$ uptake in the presence of NaCl but not NaIse. Apical membranes were vesiculated in the presence or absence of 10 μg ml^{-1} PTX, or 200 μM DTT (vehicle control), and incubated at 37°C for 1 h. AMV were then revesiculated in either 125 mM NaCl or 125 mM NaIse and allowed to equilibrate at room temperature for 1 h. The effect of PTX on conductive $^{22}Na^{+}$ uptake in the presence of NaCl or NaIse is expressed as a percentage relative to control conductive uptake levels for each anion in the presence of vehicle control. *Significantly different from vehicle control, $P < 0.05$.

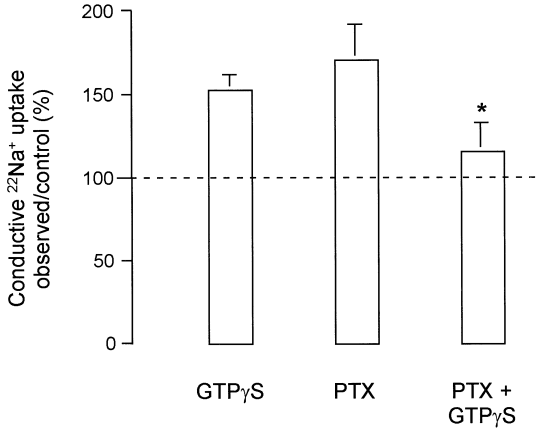

Fig. 6. GTPγS diminishes conductive $^{22}Na^{+}$ uptake in PTX pre-treated apical membrane vesicles. Apical membranes pre-treated for 1 h at 37°C with 10 μg ml^{-1} PTX or 200 μM DTT vehicle control were revesiculated in 125 mM NaCl with or without 100 μM GTPγS. The effect on conductive $^{22}Na^{+}$ uptake was calculated as a percentage relative to conductive uptake levels in the presence of vehicle control. *Significantly different from conductive uptake levels in the presence of PTX, $P < 0.05$.

3.6. Increase in $^{22}Na^{+}$ uptake in response to GTPγS is diminished in the presence of PTX

When PTX pre-treated AMV were revesiculated in the presence of GTPγS, the level of conductive $^{22}Na^{+}$ uptake was diminished (Fig. 6). Since GTPγS did not stimulate uptake after PTX treatment, we may draw the conclusion that both pathways utilise PTX-sensitive G proteins. However, as the combined effect was significantly lower compared to that of PTX alone ($P \leq 0.05$) but not control ($P = 0.4$), we can infer that

there may be additional PTX-insensitive G proteins modulating conductive $^{22}Na^+$ uptake in these AMV but there are other possibilities (see Section 4).

3.7. Several PTX-sensitive and PTX-insensitive G proteins are present in the late gestation fetal guinea pig distal lung apical membrane

Optimal PTX-dependent ribosylation of AMV proteins was achieved after 1 h at 37°C using [^{32}P]NAD as a substrate and predominantly labelled proteins of approx. 38–42 kDa in all samples (Fig. 7). Several other proteins were also labelled, two of which have previously been described in lung [19].

Further resolution of the G proteins by Western blotting of apical membrane revealed the presence of several PTX-sensitive isoforms; the 40–41 kDa proteins of $G_i\alpha_1$, $G_i\alpha_2$ and $G_i\alpha_3$, and the 39 kDa isoforms of $G_o\alpha_1$ and $G_o\alpha_2$. The level of immunostaining of $G_i\alpha_2$ was higher than the other PTX-sensitive G proteins consistent with its abundance in these preparations. In addition, we also demonstrated the presence of the 42–43 kDa PTX-insensitive G protein isoforms $G_q\alpha$ and $G_{11}\alpha$ (Fig. 8).

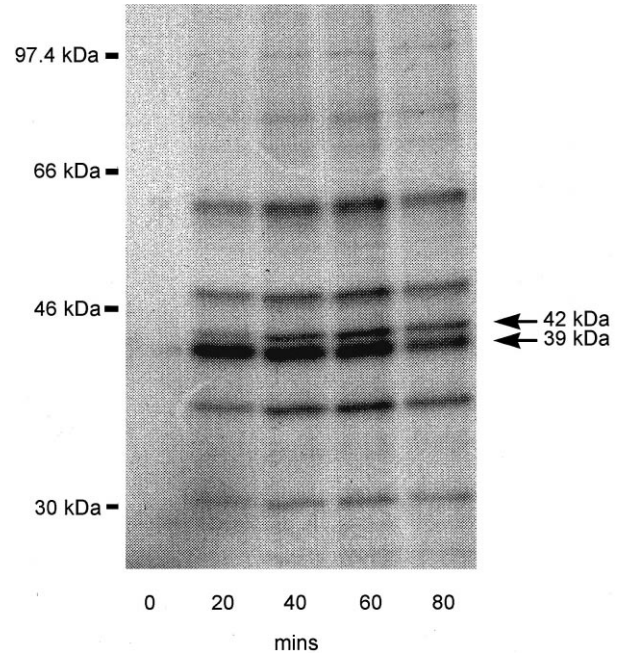

Fig. 7. Time course of PTX-dependent ADP-ribosylation of apical membrane proteins. Apical membranes were incubated with 10 μg ml^{-1} PTX, in the presence of [^{32}P]NAD and incubated at 37°C for 1 h. Labelled protein samples of 30 μg were resolved by SDS-PAGE on a 10% acrylamide, 0.26% bis gel. The ADP-ribosylated PTX-sensitive G proteins are those labelled approx. 39 and 42 kDa.

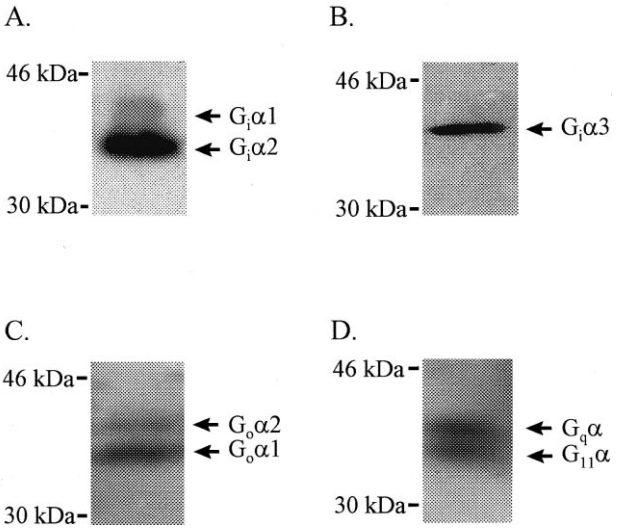

Fig. 8. PTX-sensitive and PTX-insensitive G proteins are present in lung apical membrane. Western blotting of G protein α-subunits in apical membrane from late gestation fetal guinea pig lung identified both PTX-sensitive and PTX-insensitive G protein isoforms. (A) $G_i\alpha_{1/2}$; (B) $G_i\alpha_3$; (C) $G_o\alpha_{1/2}$; (D) $G_{q/11}\alpha$. $G_i\alpha_2$ was the predominant immunostained PTX-sensitive isoform in these preparations.

4. Discussion

4.1. Conductive $^{22}Na^+$ uptake

The apical membrane vesicle system used here has been previously characterised by Fyfe et al. [7] and established as a model to investigate conductive Na^+ transport through apically located channels. The driving force for $^{22}Na^+$ uptake is created by the establishment of an electrical diffusion potential that amplifies the movement of Na^+ into the vesicles via conductive pathways. The magnitude of the diffusion potential is a function of the externally directed chemical Na^+ gradient and the relative permeability of the apical plasma membrane to anions (see Section 2). Thus, both activation of Na^+ channels and a reduction in anion permeability will increase vesicular $^{22}Na^+$ uptake and vice versa. We have demonstrated that the intravesicular substitution of a relatively impermeant anion Ise^-, or blockade of Cl^- channels with extravesicular application of the stil-

bene anion channel blocker SITS, increases conductive $^{22}Na^+$ uptake. These effects suggest that AMV possess a significant Cl^- conductance – a potential target for regulation by G proteins. However, it could be argued that the process of lowering intravesicular Cl^- might activate a cation conductance, either directly or via G protein modulation. Whilst there is convincing evidence from whole cell patch clamp studies in rat fetal distal lung epithelial cells (FDLE) [22] and mouse mandibular duct cells [23] demonstrating activation of a cation conductance as a result of Cl^- removal, the effect of reducing ambient Cl^- on G protein activity is inhibitory [24]. Since we know that the effect of G protein inhibition with GDPβS is to reduce conductive Na^+ uptake into AMV, and substitution of Cl^- by Ise^- stimulates uptake, it seems unlikely that the effects of Ise^- are due to release of a G protein mediated Cl^- inhibitory effect on the Na^+ conductive pathway.

The possibility that a reduction in ambient Cl^- might initiate a non-G protein mediated effect on apically located cation channels warrants further examination. Nevertheless, we think this an unlikely explanation for the observed effects of substituting Cl^- with Ise^- for a number of reasons. (1) The Cl^- inhibitory effect on whole cell cation currents in FDLE is dependent on $[Ca^{2+}]_i$ in the micromolar range, whereas our uptake measurements are undertaken in nominally Ca^{2+}-free conditions. (2) In rat salivary gland duct cells Cl^-, but not I^-, suppresses cation currents whereas we find that NaCl and NaI are equally effective in driving conductive $^{22}Na^+$ uptake [25]. (3) In guinea pig ATII cells we have not observed activation of whole cell cation currents when NaCl is substituted by symmetrical Na glutamate (unpublished observations). (4) Addition of the stilbene anion channel inhibitor, SITS, to AMV in which the outwardly directed gradient is established by NaCl, stimulates $^{22}Na^+$ uptake to levels equivalent to those seen in the presence of NaIse. Although it could be argued that this effect on conductive Na^+ uptake might be the result of the known ability of SITS to stimulate non-specific cation channels [26], this stimulatory effect of SITS only occurs in the presence of millimolar Ca^{2+} and requires cytosolic application of SITS, neither of which pertain in our experiments. Furthermore, we have not seen activation of whole cell cation currents by SITS in fetal guinea pig ATII cells (unpublished observations). (5) In single channel studies it can be demonstrated that the guinea pig ATII cells possess a large conductance Cl^- channel which is inhibited by bath application of 100 μM SITS [27].

For the reasons given above, and in the absence of any data suggesting that isethionate can activate cation channels, we interpret the data from Ise^- and SITS experiments as demonstrating the presence of an anion conductance in AMV. Accordingly, we have compared the results of experiments in which NaCl and NaIse are used to generate electrical diffusion gradients to drive $^{22}Na^+$ uptake in order to differentiate between G protein modulation of Na^+ and Cl^- conductive pathways.

4.2. *Na^+ and Cl^- channels of late gestation fetal guinea pig distal lung AMV*

Data from a previous study of late gestation fetal guinea pig lung AMV [7], together with measurements from excised patches of late gestation fetal ATII cells [14], suggest that the Na^+ channels of these AMV predominantly fall into the category of L-type channels exhibiting low amiloride affinity, low Na^+/K^+ selectivity [28] and are likely to comprise of, at least, the translation product of αENaC [16]. The identity of the Cl^- channels present in the AMV is more elusive. Our observations imply that there is a Cl^- conductance in the fetal AMV that is relatively permeable to Cl^- and blockable with SITS. This could be explained by the presence of the large conductance Cl^- channel we have functionally characterised by patch clamp in guinea pig ATII cells of similar developmental age [27]. Other candidates include the volume activated Cl^- channel, ClC-2, which has also been demonstrated in the apical membrane of fetal rat lung by Western blotting [29] and CFTR. Although mRNA coding for CFTR can be detected in mid-trimester human fetal lung, levels decline, particularly in the alveolus, near term [30,31]. The activity of this channel, which is not blockable by DIDS and therefore unlikely to be blockable with SITS, also declines towards term to almost undetectable levels in the adult ATII cell [32,33].

4.3. G protein regulation of Na^+ and Cl^- conductances in late gestation guinea pig lung AMV

Our demonstration that GTPγS inhibits Cl^- conductance and also stimulates Na^+ conductance in these AMV, extends our previous observations that co-localised G proteins regulate amiloride-sensitive sodium channels [14,16]. Unlike renal epithelium, in which amiloride-sensitive channels are modulated by ADP-ribosylation with PTX [34,35], we find that the predominant effect of PTX is via an inhibition of Cl^- channels. Our findings are similar to those of Berdiev et al. [36] who also found that PTX treatment did not significantly affect $^{22}Na^+$ uptake of rabbit adult ATII cells or activity of the reconstituted channel complex in bilayers. However, our observation that GTPγS stimulates conductive $^{22}Na^+$ uptake through amiloride-sensitive channels both in the AMV studied here and in excised patches [14,16], contrasts with the findings of Berdiev et al. and may be indicative of age-dependent regulatory changes and/or species differences. The fact that GTPγS also inhibited the Cl^- conductance is consistent with our previous observation that in excised patches from fetal ATII cells, GTPγS inhibits the large conductance Cl^- channel via a voltage-dependent decrease in Po [27].

4.4. G protein identity

What is the identity of the G proteins involved in the regulation of Na^+ and Cl^- channels in these AMV? PTX ribosylation effectively uncouples G_i and G_o proteins from their receptors, preventing dissociation of the heterotrimer. Consequently, the PTX mediated effect on Cl^- conductance is distinct from that of GTPγS (which locks dissociated α-subunits in their activated state) and must utilise a PTX-sensitive G protein that tonically activates Cl^- channels under resting conditions. Since GTPγS did not augment the effect of PTX it appears that the GTPγS mediated stimulation of Na^+ channels and inhibition of Cl^- channels also utilises a PTX-sensitive G protein(s).

The fact that intravesicular incorporation of GTPγS after PTX treatment diminishes conductive $^{22}Na^+$ uptake to a level not significantly different from control raises several possibilities. Firstly, PTX ribosylation may unmask a PTX-insensitive G protein that inhibits Na^+ channels and/or stimulates Cl^- channels. This generates a more complex regulatory model involving three G proteins but is consistent with the demonstration that a significant component of high affinity GTPase activity in these AMV is PTX-insensitive [37]. Secondly, the effect of GTPγS and PTX may be dependent on the activation state of the channel. This final possibility has been described for the regulation of Na^+ channels in lymphocytes [38] and renal epithelium [35]. In our system, it may be possible that such regulatory pathways exist for the control of Cl^- channels.

We have confirmed specific PTX-dependent ribosylation of the G protein isoforms G_i and G_o in apical membrane and PTX treatment causes similar inhibition of GTPase activity in F1–3 AMV [37]. Western blotting has further resolved the presence of $G_i\alpha_1$, $G_i\alpha_2$ and $G_i\alpha_3$ in addition to $G_o\alpha_1$ and $G_o\alpha_2$. Although several PTX-sensitive G proteins are present in the AMV, the specific identities of the G protein isoforms involved in the regulatory pathways we describe remain elusive. The demonstration that purified recombinant $G_i\alpha_3$ increases amiloride-sensitive sodium channel activity in excised patches from A6 cells [33], and is co-localised with the channel complex in kidney [13], is consistent with our findings but this particular isoform has not been demonstrated as part of the amiloride-sensitive sodium channel complex of rabbit ATII cells [39]. Thus, there here must be some doubt as to whether $G_i\alpha_3$ is involved in GTPγS stimulation of Na^+ transport in lung. Furthermore the phospholipase A2 pathway to which it is linked in A6 cells is apparently absent in ATII cell apical membranes [16]. The high level of expression of $G_i\alpha_2$, that we and others describe in the alveolar epithelium [40] and apical membrane preparations, suggests that it may have an important regulatory role. However, in relation to Na^+ conductance, our findings are unlike those in adult rabbit ATII cells where $G_i\alpha_2$ has been shown to inhibit amiloride-sensitive epithelial sodium channels. This could be due to gestational age or species differences between the two preparations [36]. $G_i\alpha_2$ has also been implicated with $G_i\alpha_1$ to modulate epithelial Na^+ channels in response to changes in intracellular Cl^- concentration in mouse salivary duct cells [41]. However, there is no evidence to suggest that this regulatory mechanism operates in guinea pig ATII cells (see also Section 4.1). As re-

gards a role for $G_i\alpha_2$ in the regulation of Cl^- conductance, this isoform has been shown to inhibit outwardly rectifying Cl^- channels in airway [42]. However, even though activation of apically located G proteins has been shown to inhibit a large conductance Cl^- channel in guinea pig ATII cells [27] there is no evidence specifically implicating involvement of $G_i\alpha_2$ in this process.

Possible roles for the PTX-sensitive G protein $G_o\alpha$ are more elusive. $G_o\alpha$ has been shown to mediate an inhibitory effect of cytosolic Na^+ on apical Na^+ channels in salivary gland [41] but it is not known whether this regulatory mechanism exists in alveolar epithelium.

The G protein isoforms $G_q\alpha$ and $G_{11}\alpha$ are also present in the AMV and may be involved in the PTX-insensitive inhibition of Na^+ channels and/or stimulation of Cl^- channels. However, evidence suggests that these G proteins mediate their effects through the phospholipase C modulation of intracellular Ca^{2+}, a mode of action unlikely in our AMV due to the absence of such cytosolic components.

4.5. Mode of action of G proteins upon channels

How do these G proteins exert their effect on the Na^+ and Cl^- channels in the AMV? In the absence of cytosolic second messengers these effects are likely to be a direct action of the G protein upon the channels [43] and this is supported by our observations that DTT did not inhibit the ability of GTPγS to stimulate conductive $^{22}Na^+$ uptake. This is in contrast to renal epithelium where dissociation of proteins from the sodium channel complex with DTT prevented G protein mediated protein kinase A regulation [21]. It is certainly true that fewer associated proteins have been identified in the ATII cell than the renal sodium channel complex by immunoprecipitation [3].

Our finding that DTT inhibited conductive $^{22}Na^+$ uptake in vesicles containing NaCl or NaIse raises the possibility that thiol reduction alters the conductance state of Na^+ channels by modification of cytoskeletal proteins that are associated with the channel [44], [45], [46]. Actin is certainly present in the apical membrane preparation (L. Gambling, D.L. Baines, unpublished observations). Alternatively, DTT may have a direct action on the channel subunits. When renal amiloride-sensitive sodium channels are reconstituted into bilayers, DTT primarily reduces the conductance of channels formed by αENaC rather than α,β,γENaC [47]. We have demonstrated by Northern blotting and RT-PCR that αENaC mRNA is predominantly expressed over βENaC in guinea pig distal lung and ATII cell preparations at this time [48]. We have not specifically investigated the expression of γENaC in our system but by analogy with other studies we expect its ontogeny to follow closely that of βENaC [49]. Thus, we postulate that the effect of DTT in these AMV, taken together with the relatively high conductance of the channel we describe in ATII cells [14], may indicate a predominant expression of channels formed by αENaC alone in fetal guinea pig AMV [50]. Certainly the importance of αENaC in fluid absorption at birth [51] is undisputed.

In conclusion, we have identified a number of apically located PTX-sensitive and -insensitive G proteins and have confirmed that conductive $^{22}Na^+$ uptake in fetal distal lung AMV is stimulated by GTPγS. We propose that transduction of this pathway is via activation of one or more apically co-localised PTX-sensitive G proteins which directly activate amiloride-sensitive sodium channels and concurrently inactivates apical Cl^- channels. PTX also stimulates conductive $^{22}Na^+$ uptake in these AMV via an inhibition of Cl^- channels. Understanding these apically localised regulatory pathways may be important in the elucidation of factors that regulate the absorption of lung fluid in preparation for birth.

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