P₂-purinoceptor regulation of surfactant phosphatidylcholine secretion

Relative roles of calcium and protein kinase C

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Ca²⁺ and protein kinase C have both been proposed as intracellular signals for subsequent phosphatidylcholine secretion by alveolar Type II cells. We have determined the relative roles of Ca²⁺ and protein kinase C in regulating surfactant phosphatidylcholine secretion by utilizing exogenous ATP and the phorbol ester TPA (12-*O*-tetradecanoylphorbol 13-acetate) as secretagogues, along with MAPTAM to chelate intracellular Ca²⁺ and sphingosine to inhibit endogenous protein kinase C. Exposure of Type II cells to the P₂-purinoceptor agonist, ATP, results in a dose-dependent increase in surfactant phosphatidylcholine secretion from isolated alveolar Type II cells with an EC₅₀ (concn. producing 50% of maximal response) of 2 μM. Administration of exogenous ATP to Type II cells also results in a dose-dependent increase in inositol trisphosphate production, Ca²⁺ mobilization and [³H]phorbol 12,13-dibutyrate ([³H]PDBu) binding as a measure of protein kinase C translocation. The EC₅₀ in each case is 1–5 μM, indicating association of these events with surfactant phosphatidylcholine secretion. Loading Type II cells with a non-hydrolysable GTP analogue (GTP[S]) inhibited ATP-induced Ca²⁺ mobilization, supporting the hypothesis that Type II cell P₂-purinoceptors are coupled to phospholipase C via a GTP-binding protein. The ATP-induced elevation of cytosolic Ca²⁺ was also inhibited by MAPTAM (a cell-permeant EGTA analogue) by 90%, but MAPTAM was without effect on surfactant phosphatidylcholine secretion induced by ATP. Sphingosine inhibited both ATP- and TPA-induced surfactant phosphatidylcholine secretion as well as [³H]PDBu binding with a similar IC₅₀ (concn. producing 50% of maximal inhibition) (10 μM). Sphingosine did not affect surfactant phosphatidylcholine secretion induced by terbutaline and did not have a significant effect on Ca²⁺ mobilization induced by exogenous ATP. These results are consistent with a prominent role for protein kinase C in regulation of P₂-purinoceptor-induced surfactant phosphatidylcholine secretion, and indicate that Ca²⁺ mobilization is not a necessary step for ATP-induced surfactant phosphatidylcholine secretion.

INTRODUCTION

Elevated intracellular Ca²⁺ functions as a second messenger, regulating secretion in many cell types, including the alveolar Type II cell of the lung which is responsible for production and secretion of surfactant necessary for maintenance of normal lung function [1–3]. Previous work with ionophores demonstrated that elevated Ca²⁺ levels are capable of augmenting surfactant phosphatidylcholine (PC) secretion [1–3], but the steps following Ca²⁺ mobilization which mediate surfactant secretion remain unclear. Until the discovery of the Type II cell P₂-purinoceptor which binds exogenous ATP [4,5], a physiological receptor coupled to elevation of intracellular Ca²⁺ levels in Type II cells was lacking. Exogenous ATP is one of the most potent stimuli for surfactant PC secretion presently available which mobilizes intracellular stores of Ca²⁺ [6]. Previous work demonstrated an association between the rank order of different P₂-agonists for Ca²⁺ mobilization and the rank order of P₂-agonists for surfactant secretion for a series

of ATP analogues [6]. In addition, the dose response for inositol trisphosphate (IP₃) production in response to exogenous ATP resembles the dose response for ATP-induced surfactant secretion [7], although the steps following Ca²⁺ mobilization which mediate surfactant secretion again remain unclear. It is also unclear whether ATP functions to regulate surfactant secretion *in vivo*, although ATP is present in bronchoalveolar lavage fluid in quantities sufficient to mediate surfactant PC secretion *in vivo*, when compared with results *in vitro* [8].

P₂-purinoceptors which bind exogenous ATP appear to belong to the class of receptors coupled to phospholipase C via GTP-binding proteins [9–12], although this has not been demonstrated directly for the Type II cell. Thus, in addition to mobilization of intracellular Ca²⁺, diacylglycerol (DAG) generation may activate protein kinase C in Type II cells exposed to exogenous ATP as well, although this has not been previously shown. In astrocytoma cells, 100 nM-ionomycin causes translocation with subsequent activation of protein kinase C as well as Ca²⁺ mobilization

Abbreviations used: PC, phosphatidylcholine; PDBu, phorbol 12,13-dibutyrate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; IP₃, inositol trisphosphate; EC₅₀, concentration producing 50% of the maximal response; DAG, diacylglycerol; GTP[S], guanosine 5'-[γ-thio]triphosphate; GDP[S], guanosine 5'-[β-thio]diphosphate; IC₅₀, concentration producing 50% of the maximal inhibition; MAPTAM, bis-(2-amino-5-methylphenoxy)ethane-*NNN'*-tetra-acetic acid tetra-acetoxymethyl ester.

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[13]. Since the same dose of this Ca^{2+} ionophore causes Ca^{2+} mobilization in Type II cells in association with surfactant phospholipid secretion [2,3], we hypothesized that ionomycin-induced surfactant secretion is mediated by protein kinase C, and tested whether ATP-induced surfactant secretion requires prior mobilization of intracellular Ca^{2+} . We also examined the relative roles of Ca^{2+} and protein kinase C in regulating surfactant PC secretion. Previous work with isolated alveolar Type II cells has demonstrated augmented surfactant secretion and protein kinase C translocation in response to the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA), but the synthetic DAG analogue oleoylacylglycerol did not have a similar effect on protein kinase C translocation, although this analogue did induce surfactant PC secretion [14].

Since purinoceptor-induced protein kinase C translocation has not been demonstrated in any cell type, and it is unclear from previous work whether DAG generation in alveolar Type II cells results in protein kinase C translocation, we addressed these questions in the present work.

EXPERIMENTAL

Materials

[$\text{Me-}^3\text{H}$]Choline chloride, dipalmitoyl [^{14}C]PC and [^3H]phorbol 12,13-dibutyrate ([^3H]PDBu) were purchased from New England Nuclear (Boston, MA, U.S.A.). Chloroform, methanol and Scintiverse II were from Fisher Chemical Co. (Cincinnati, OH, U.S.A.). D-Sphingosine, PDBu, GTP and guanosine 5'-[β -thio]diphosphate (GDP[S]) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Guanosine 5'-[γ -thio]triphosphate (GTP[S]) was from Boehringer. MAPTAM was from Calbiochem Behring (La Jolla, CA, U.S.A.). Egg PC was obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Elastase was obtained from Cooper Biomedicals (Malvern, PA, U.S.A.) and Fura-2 was from Molecular Probes (Eugene, OR, U.S.A.).

Animals

Pathogen-free male Sprague-Dawley rats (200–250 g) were obtained from Charles River (Wilmington, MA, U.S.A.).

Isolation and culture of Type II cells

Type II cells were isolated from rat lungs by a modification of the method described previously [15]. Rats were maintained under sterile guard hooded cages and allowed food and water *ad libitum* before the experiment. Rats were then anaesthetized with sodium pentobarbitone and their lungs were perfused via the pulmonary artery with buffer A (NaCl , 125 mM; KCl , 5 mM; Na_2HPO_4 , 2.5 mM; Hepes, 17 mM; glucose, 1 mg·ml $^{-1}$; gentamycin, 10 μg ·ml $^{-1}$; penicillin, 20 units·ml $^{-1}$; pH 7.4) containing 3% (w/v) bovine serum albumin. Lungs were then lavaged as described previously, and incubated with elastase solution (40 ml; 0.4 units·ml $^{-1}$ in buffer A) for 20 min at 37 °C. Tissue was minced and filtered through progressively smaller Nitex gauze as described, and washed in buffer A. Cells were then resuspended in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, U.S.A.) and incubated on 100 mm bacteriologic plastic Petri dishes which had been precoated with 500 μg of IgG·ml $^{-1}$ for

3 h at room temperature. Following a 1 h incubation at 37 °C in air/ CO_2 (19:1), the plates were 'panned' as described and the unattached cells were removed and collected by centrifugation. Isolated cells were resuspended in Dulbecco's modified Eagle's medium containing 10% foetal calf serum and utilized for experiments following 18 h in culture at 37 °C in air/ CO_2 (19:1). This procedure routinely yielded 40×10^6 cells/rat. For release experiments, cells were diluted in plating medium to 0.5×10^6 cells·ml $^{-1}$. Cell suspension (1 ml) was placed in each well of a 24 well tissue culture plate which had been precoated with collagen (Collaborative Research, Lexington, MA, U.S.A.) and prelabelled by addition of 1 μCi of [^3H]choline (sp. radioactivity 80.0 Ci·mmol $^{-1}$). The plating efficiency at 18 h was generally 60–70%. Non-adherent cells were removed from the wells by washing before the assay. Cultures contained $89 \pm 2\%$ viable Type II cells as determined by fluorescence staining with Phosphine 3R.

Secretion of PC

Secretion of PC by cultured Type II cells was determined as described previously [5]. Briefly, cells incubated overnight were washed three times with Dulbecco's modified Eagle's medium containing 40 mM-Hepes and 3 mg of bovine serum albumin·ml $^{-1}$, pH 7.4, at 37 °C (buffer B). Cells were allowed to equilibrate for 30 min and at the end of this time, agents were added and the [^3H]PC released was determined after 3 h. Medium was aspirated and the cells were washed with 0.5 ml of fresh medium. The two samples of medium were then combined and centrifuged at 9000 *g* for 5 min to pellet the cells. Supernatant was removed and lipid extracted according to Folch, with addition of 1 mg of egg PC as a carrier.

Cells remaining in the wells were extracted with 2×1 ml of ethanol and fractions were obtained as for the medium. Samples containing lipid were dried overnight and radioactivity was determined with a β -scintillation counter after addition of 5.0 ml of Scintiverse II to each sample. Dipalmitoyl [^{14}C]PC was used as an internal standard to measure recoveries of [^3H]PC, which were generally $95 \pm 1\%$. The amount of [^3H]PC secretion was calculated as the percentage of total [^3H]PC present in the medium relative to the amount present in cells [i.e. c.p.m. in medium/(c.p.m. in medium + c.p.m. in cells)]. The amount of [^3H]PC released following the 30 min preincubation was subtracted from all samples.

Lactate dehydrogenase activity was determined in each sample as a measure of cytotoxicity by adding an aliquot (0.3 ml) to 0.5 ml of 250 μM -3-[*N*-morpholino]propane-sulphonic acid, pH 7.0, and 0.1 ml of freshly prepared 10 mM-NADH. The reaction was initiated by adding 0.1 ml of sodium pyruvate and the enzyme activity was determined by measuring the decrease in absorbance at 340 nm. Total activity was determined by treating sample plates with 0.1% Triton X-100. None of the agents at the concentrations utilized for these experiments resulted in statistically significant release of lactate dehydrogenase above control levels, which were 1–2% of total cellular lactate dehydrogenase released after a 3 h incubation.

Mobilization of intracellular Ca^{2+}

Intracellular Ca^{2+} levels were determined by modification of a previously published method using the fluorescent Ca^{2+} -binding dye, Fura 2 [10]. Fresh Type II

cells, or cells which had plated down overnight, were scraped from plates with a rubber policeman, spun briefly in a Microfuge (15 s at 9000 *g*), and combined in 1 ml of buffer B. Fura 2 (5 μ M) was then added to the cell suspension. Cells were incubated for 10 min at 37 °C, centrifuged at 9000 *g* for 15 s, resuspended in fresh buffer B and incubated for an additional 10 min at 37 °C. Loaded cells were then washed three times with ice-cold buffer B to remove extracellular dye and stored on ice before use. For fluorescence experiments, 4×10^6 cells were resuspended in 1 ml of buffer B and incubated at 37 °C. Fluorescence was monitored using an excitation wavelength of 339 nm and an emission wavelength of 500 nm. Each sample was standardized after cell lysis with Triton X-100 (0.1 %). The fluorescence maximum was determined in the presence of 1 mM- Ca^{2+} and the fluorescence minimum in the presence of alkaline 2 mM-EGTA. Cell autofluorescence was determined using unloaded cells and the quantity of extracellular dye present was determined in the presence and absence of extracellular EGTA (1 mM). Utilizing this protocol, cells contained 10–20 μ M-Fura 2 and leakage of dye was $<5\% \cdot \text{h}^{-1}$. Cells were also uniformly labelled when examined by fluorescence microscopy without evidence of the organellar sequestration which has been reported with longer incubation times [2]. For experiments involving MAPTAM, this agent was loaded into cells at the concentrations noted as described previously [16] for 30 min before addition of agonists.

Measurement of IP_3 and DAG

Isolated alveolar Type II cells were labelled for 18 h with [^3H]inositol using inositol-free media or

[^3H]arachidonic acid. Cells were then washed three times with fresh medium and incubated for 30 min in medium containing 10 mM-LiCl (for IP_3) or regular media (DAG). At the end of this time, ATP or buffer was added for the noted time. The extraction and purification of IP_3 and DAG were as described previously [10].

[^3H]PDBu binding to intact cells

Type II cells were plated overnight in 24 well plates (approx. 1×10^6 cells/well) as described above. They were washed three times and preincubated for 30 min at 37 °C in fresh medium. At the end of this time, medium was removed and replaced with fresh medium containing 20 nM-[^3H]PDBu for 5 min at 37 °C as described by Trilivas & Brown [13]. ATP or buffer was then added for the desired time period, after which cells were washed rapidly three times with fresh medium and 0.5 ml of 1 M-NaOH was then added. Non-specific binding was determined in the presence of 10 μ M-PDBu. Samples were neutralized with 0.5 ml of 1 M-HCl and specific binding was determined as described previously [13].

Scrape loading of Type II cells with GTP analogues

Type II cells were plated overnight in collagen-coated 100 mm Petri dishes. The cells were washed three times and 1 ml of fresh medium containing the desired agent was added. Cells were scraped from the dish using a rubber policeman as described previously [17] and loaded with Fura 2 as noted above.

Statistical analyses

Kruskal-Wallis one-way analysis of variance by ranks [18] or Friedman two-way analysis of variance by ranks

Fig. 1. Dose-response curves for ATP-stimulated [^3H]PC secretion (a), Ca^{2+} mobilization (b), IP_3 production (c) and [^3H]PDBu binding (d)

Type II cells were obtained as stated in the Experimental section and plated for 18–20 h in primary culture in the presence of [^3H]choline (a), [^3H]inositol (c) or buffer (b and d). Assays were performed as stated in the Experimental section after exposure to ATP for 3 h (a), 10 s (b), 2 min (c) or 5 min (d). Data represent the means $\pm$ S.E.M. for four (a) or five (b–d) experiments, each in duplicate to quadruplicate.

for non-parametric data [19] was used as appropriate. Analysis of variance for both parametric and non-parametric data was performed on an IBM PC XT microcomputer using commercially available statistical packages.

RESULTS

Exogenous ATP stimulates surfactant phospholipid secretion from isolated Type II cells in a dose-dependent fashion with an EC_{50} (concn. producing 50% of maximal effect) of $2 \mu M$ (Fig. 1). Exogenous ATP also causes a time-dependent increase in IP_3 production, DAG production and [3H]PDBu binding activity, which peaks 2–5 min after exposure to ATP and returns to basal levels by 15 min after exposure (Fig. 2). The dose responses for IP_3 production and [3H]PDBu binding were similar to that for surfactant phospholipid secretion, each with an EC_{50} of 1–5 μM (Fig. 1). We also determined peak intracellular Ca^{2+} levels in Type II cells after

Fig. 2. Time course for ATP-stimulated phosphatidylinositol turnover

Type II cells were prepared as stated in the Experimental section before determination of IP_3 production (a), DAG production (b) or PDBu binding (c) following exposure to ATP ($10 \mu M$) for the times noted. Data represent the means $\pm$ S.E.M. for three to six experiments.

Fig. 3. Effect of scrape-loading GTP analogues on ATP-induced Ca^{2+} mobilization

Type II cells plated for 18 h as described in the Experimental section were scrape-loaded in the presence of buffer (control), 2 mM-GDP[S] or 2 mM-GTP[S] as noted. Cells were then loaded with Fura 2 as described and ATP ($10 \mu M$) was added at the break in the tracing. Tracings are representative from one of three such experiments.

exposure to exogenous ATP (Fig. 1), and found an EC_{50} of $1 \mu M$. The maximal effect of ATP occurred at 10–100 μM for all four processes (Fig. 1).

These data provide evidence for phospholipase C involvement in ATP-induced phosphatidylinositol hydrolysis in Type II cells. Since other receptors are coupled to phospholipase C via a GTP-binding protein, we tested this more directly for the Type II cell P_2 -purinoceptor by scrape loading Type II cells in the presence of buffer, GDP[S] or GTP[S] (Fig. 3; Table 1). Cells loaded with GTP[S] demonstrated an attenuated mobilization of intracellular Ca^{2+} in response to exogenous ATP relative to control cells or cells exposed to GDP[S], supporting the presence of a GTP-binding protein in alveolar Type II cells which couples ATP binding to Ca^{2+} mobilization.

Although dose-response curves for surfactant PC secretion, Ca^{2+} mobilization, IP_3 production and PDBu

Fig. 4. Effect of MAPTAM on ATP-induced [3H]PC release and Ca^{2+} mobilization

Type II cells prepared as stated in the Experimental section were treated with the indicated concentrations of MAPTAM for 30 min before addition of ATP ($10 \mu M$) for 3 h (a) or 10 s (b). Data represent means $\pm$ S.E.M. for six experiments utilizing 18 h-plated cells (a) or fresh cells (b) MAPTAM had no statistically significant effect on ATP-induced [3H]PC secretion, but did inhibit Ca^{2+} mobilization.

Table 1. Effect of scrape loading Type II cells with GTP analogues on ATP-induced Ca^{2+} mobilization

Type II cells plated for 18 h were scrape-loaded in buffer containing no additions (control), GDP[S] (2 mM) or GTP[S] (2 mM), and then loaded with Fura-2 as described in the Experimental section. Results are means $\pm$ S.E.M. of three experiments, each in duplicate or triplicate. GTP[S] had a significant effect ($P < 0.05$) on ATP-induced Ca^{2+} mobilization, whereas basal levels of Ca^{2+} were not significantly different.

Treatment	Basal $[Ca^{2+}]_i$ (nM)	Peak change in $[Ca^{2+}]_i$ (nM)
Control	259 ± 26	136 ± 23
GDP[S]	275 ± 21	131 ± 14
GTP[S]	276 ± 18	55 ± 11

binding were similar (Fig. 1), whether Ca^{2+} mobilization, protein kinase C activation or both are necessary for ATP-induced surfactant PC secretion is not known. Therefore we determined the relative roles of Ca^{2+} mobilization and protein kinase C activation in the regulation of surfactant PC secretion. We first attempted to block ATP-induced surfactant PC secretion using a

Fig. 5. Effects of sphingosine on (a) ATP-, (b) TPA- and (c) terbutaline-induced $[^3\text{H}]\text{PC}$ secretion

Type II cells were exposed to ATP ($10\ \mu\text{M}$), TPA ($0.1\ \mu\text{M}$) or terbutaline ($10\ \mu\text{M}$) for 3 h and $[^3\text{H}]\text{PC}$ release was determined. Data are from three experiments, each in triplicate. Sphingosine was present for 30 min before agonist addition and significantly inhibited ATP- and TPA-induced $[^3\text{H}]\text{PC}$ release, but did not affect terbutaline-induced $[^3\text{H}]\text{PC}$ release.

Fig. 6. Effect of sphingosine on ATP-induced Ca^{2+} mobilization and $[^3\text{H}]\text{PC}$ release

Fresh (a) or 18 h (b) Type II cells were exposed to sphingosine for 30 min and Ca^{2+} mobilization or $[^3\text{H}]\text{PDBu}$ binding was measured as noted in the Experimental section following exposure to ATP ($10\ \mu\text{M}$) for 10 s (a) or 5 min (b). Sphingosine significantly inhibited ATP-induced $[^3\text{H}]\text{PDBu}$ binding, but did not have a significant effect on ATP-induced Ca^{2+} mobilization.

membrane-permeant EGTA analogue, MAPTAM. Although MAPTAM markedly inhibited Ca^{2+} mobilization in response to exogenous ATP, it was without significant effect on surfactant PC secretion (Fig. 4), implicating protein kinase C, as opposed to Ca^{2+} mobilization, as a key mediator of ATP-induced surfactant PC secretion.

We next tested whether sphingosine, a protein kinase C antagonist, could inhibit ATP-induced surfactant phospholipid secretion. Sphingosine markedly inhibited surfactant PC secretion that was induced by exogenous ATP or the phorbol ester TPA, but was without effect on β -agonist-induced surfactant PC secretion (Fig. 5). Sphingosine also inhibited $[^3\text{H}]\text{PDBu}$ binding induced by ATP (Fig. 6) and the IC_{50} (concn. producing 50% of maximal inhibition) for sphingosine-induced inhibition of ATP-generated $[^3\text{H}]\text{PC}$ secretion was similar to the IC_{50} for inhibition of $[^3\text{H}]\text{PDBu}$ binding ($10\ \mu\text{M}$). However, sphingosine was without effect on Ca^{2+} mobilization in the Type II cells (Fig. 6), implicating protein kinase C as the key factor mediating ATP-induced surfactant phospholipid secretion.

DISCUSSION

Exogenous ATP is a potent stimulus for surfactant PC secretion in association with IP_3 production, intracellular Ca^{2+} mobilization, DAG generation and increased PDBu binding. The EC_{50} for all these events is similar, indicating association of ATP binding to P_2 -purinoceptors in the Type II cell with all of these processes.

The role of Ca^{2+} in the regulation of surfactant PC secretion by Type II cells was investigated previously with Ca^{2+} ionophores A23187 and ionomycin [1–3]. Both of these ionophores stimulated surfactant phospholipid secretion. Although the present data would appear to contradict these earlier results, it is possible that the effect of Ca^{2+} in the previous studies was mediated via protein kinase C. This hypothesis is supported by data demonstrating that ionomycin causes protein kinase C translocation in astrocytoma cells [13], but remains to be demonstrated directly in the Type II cell. Certainly, from the present study, Ca^{2+} mobilization would appear to play a less important role in regulation of ATP-induced surfactant PC secretion, relative to the role played by protein kinase C. However, basal levels of Ca^{2+} are probably required for efficient secretion as demonstrated previously [12], and MAPTAM had no effect on basal Ca^{2+} levels under the present conditions.

A recent report indicates that sphingosine may not be specific for protein kinase C and may also inhibit Ca^{2+} /calmodulin-dependent protein kinase [20]. This would reconcile the present data with previous data using Ca^{2+} ionophores as secretagogues for surfactant PC secretion from Type II cells [1–3]. However, the data from the present study with MAPTAM would argue against involvement of Ca^{2+} /calmodulin-dependent kinase in ATP-mediated surfactant secretion, since MAPTAM blocks Ca^{2+} mobilization without affecting surfactant PC secretion. In addition, sphingosine blocked $[^3\text{H}]\text{PDBu}$ binding in the present experiments in a dose-dependent manner analogous to the effect of sphingosine on surfactant secretion, supporting protein kinase C and not Ca^{2+} /calmodulin-dependent kinase as a mediator of ATP-induced surfactant PC secretion.

The present data are consistent with previous observations demonstrating protein kinase C trans-

location in association with TPA-induced PC secretion from alveolar Type II cells [14]. Although protein kinase C translocation was observed in that study in response to TPA, oleoylacetylgllycerol treatment did not result in a similar translocation. Assessing protein kinase C translocation by measuring changes in PDBu binding offers distinct advantages over previous techniques. Subcellular fractionation of cells is not necessary, reducing the introduction of experimental artifacts [21]. It is also possible to examine effects in intact cells at early time points using small numbers of cells. In previous work with Type II cells, the effect of oleoylacetylgllycerol was examined at 30 min because the technique used necessitated recovery of protein kinase C activity from cell fractions. ATP-enhanced [3 H]PDBu binding at early time points in the present study, and an effect of oleoylacetylgllycerol on protein kinase C translocation, may not have been appreciated at earlier time points in the previous study [14]. Our work provides further support for the technique developed by Trilivas & Brown [13] for examining protein kinase C translocation in intact cells. Because of the smaller number of cells required, this method should be applicable to other systems where an examination of protein kinase C translocation has heretofore not been possible.

Our work also demonstrates for the first time that alveolar Type II cells can be scrape-loaded successfully in the presence of low-molecular-mass solutes (GTP[S]) as demonstrated previously for bovine aorta endothelial cells [22] and continuous cell lines (Swiss 3T3 cells and HeLa cells) [17]. Such a procedure will help to develop a better understanding of Type II cell function, since probes which do not normally cross the membrane barrier can be admitted in this fashion to the cytosolic compartment.

Although we hypothesized that IP_3 and DAG were produced by activation of phospholipase C, the time course for IP_3 production was different from the time course for DAG production (Fig. 2). DAG levels remained elevated at 15 min post-agonist exposure, when IP_3 had returned to basal levels. Either the two second messengers may be metabolized at different rates to account for this observation, or other lipid substrates may also be hydrolysed by phospholipase C to generate a larger cellular content of DAG. Such a phenomenon was recently described for pancreatic acini stimulated by cholecystokinin [23] and astrocytoma cells stimulated by carbachol [24].

The time course we observed for DAG production in response to ATP is similar to the time course for DAG production by astrocytoma cells in response to carbachol, peaking at 5 min post-agonist exposure [13]. This time course is also consistent with a role for DAG in protein kinase C translocation in the Type II cell, since the time course for increased PDBu binding in alveolar Type II cells also peaked 5 min after exposure to ATP. This finding is distinct from data for astrocytoma cells, where increased PDBu binding preceded DAG production and was felt to result from Ca^{2+} mobilization rather than from DAG production [13]. In the Type II cells, DAG production in response to purinoceptor activation appears necessary for protein kinase C translocation, and Ca^{2+} mobilization alone may not be sufficient or required for ATP-induced protein kinase C translocation.

The results we obtained with GTP[S] loading are similar to those described previously with endothelial cells [22]. Loading cells with GTP[S] in both cases resulted

in decreased Ca^{2+} mobilization in response to the purinoceptor agonist, ATP, whereas loading either Type II cells or endothelial cells with GDP[S] was without effect [22]. This effect of GTP[S] in both cases appears to be mediated via a GTP-binding protein coupled to phospholipase C.

In summary, we have demonstrated phosphatidylinositol phosphate turnover, Ca^{2+} mobilization and increased [3 H]PDBu binding in alveolar Type II cells in association with surfactant PC secretion in response to exogenous ATP. The findings that [3 H]PDBu binding is increased after ATP exposure and that sphingosine inhibits both increased [3 H]PDBu binding and increased PC secretion support the concept that protein kinase C plays an important role in regulation of ATP-induced surfactant PC secretion, whereas Ca^{2+} mobilization alone does not account for the secretory response to exogenous ATP. Further studies to identify substrates for protein kinase C in the Type II cell should ultimately help to clarify the mechanisms regulating secretion of surfactant phospholipid.

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REFERENCES

1. Dobbs, L. G., Gonzalez, R. F., Marinari, L. A., Mescher, E. J. & Hawgood, S. (1986) *Biochim. Biophys. Acta* **877**, 305–313
2. Pian, M. S., Dobbs, L. G. & Düzgünes, N. (1988) *Biochim. Biophys. Acta* **960**, 43–53
3. Sano, K., Voelker, D. R. & Mason, R. J. (1987) *Am. J. Physiol.* **253**, C679–C686
4. Gilfillan, A. M. & Rooney, S. A. (1987) *Biochim. Biophys. Acta* **917**, 18–23
5. Rice, W. R. & Singleton, F. M. (1986) *Br. J. Pharmacol.* **89**, 485–491
6. Rice, W. R. & Singleton, F. M. (1987) *Br. J. Pharmacol.* **91**, 833–838
7. Warburton, D., Buckley, S. & Cosico, L. (1989) *J. Appl. Physiol.* **66**, 901–905
8. Rice, W. R., Burhans, M. & Wispe, J. R. (1989) *Pediatr. Res.* **25**, 396–398
9. Boyer, J. L., Downes, C. P. & Harden, T. K. (1989) *J. Biol. Chem.* **264**, 884–890
10. Dubyak, G. R. (1986) *Arch. Biochem. Biophys.* **245**, 84–95
11. Dubyak, G. R., Cowen, D. S. & Mueller, L. M. (1988) *J. Biol. Chem.* **263**, 18108–18117
12. Okajima, F., Tokumitsu, Y., Kondo, Y. & Ui, M. (1987) *J. Biol. Chem.* **262**, 13483–13490
13. Trilivas, I. & Brown, J. H. (1989) *J. Biol. Chem.* **264**, 3102–3107
14. Sano, K., Voelker, D. R. & Mason, R. J. (1985) *J. Biol. Chem.* **260**, 12725–12729
15. Dobbs, L. G., Gonzales, R. & Williams, M. C. (1986) *Am. Rev. Respir. Dis.* **134**, 141–145
16. Korchak, H. M., Vossall, L. B., Haines, K. A., Wilkenfeld, C., Lundquist, K. F. & Weissman, G. (1988) *J. Biol. Chem.* **263**, 11098–11105
17. McNeil, P. L., Murphy, R. F., Lanni, F. & Taylor, D. L. (1984) *J. Cell Biol.* **98**, 1556–1564
18. Kruskal, W. H. & Wallis, W. A. (1952) *J. Am. Stat. Assoc.* **47**, 583–621

19. Friedman, M. (1937) *J. Am. Stat. Assoc.* **32**, 675–701
20. Jefferson, A. B. & Schulman, H. (1988) *J. Biol. Chem.* **263**, 15241–15244
21. Phillips, W. A., Fujiki, T., Rossi, M. W., Korchak, H. M. & Johnston, R. B., Jr. (1989) *J. Biol. Chem.* **264**, 8361–8365
22. Brock, T. A., Dennis, P. A., Griendling, K. K., Diehl, T. S. & Davies, P. F. (1988) *Am. J. Physiol.* **255**, C667–C673
23. Matozaki, T. & Williams, J. A. (1989) *J. Biol. Chem.* **264**, 14729–14734
24. Martinson, E. A., Goldstein, D. & Brown, J. H. (1989) *J. Biol. Chem.* **264**, 14748–14754

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