Alkalosis- and ATP-induced increases in the diacylglycerol pool in alveolar type II cells are derived from phosphatidylcholine and phosphatidylinositol

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Alkalosis and ATP increase surfactant secretion in alveolar type II cells, possibly via non-receptor- and receptor-mediated mechanisms respectively. We compared the effects of these two agonists on phosphatidylinositol (PI) and 1,2-diacylglycerol (DAG) pools and on phosphatidylcholine (PC) hydrolysis in alveolar type II cells. Alkalosis, caused by transfer of cells from 5% (control) to 0% CO₂ in air, and ATP increased the secretion of surfactant compared with the controls. The stimulated secretion was inhibited by staurosporine, a protein kinase C inhibitor. DAG and PI contents of control cells were 50 ± 1.1 (means ± S.E.M., *n* = 8) and 14 ± 0.8 nmol/mg phospholipid (*n* = 7) respectively. The DAG content increased by ~ 50 nmol (100%) within 5 s of treatment with both alkalosis and ATP, returned to control levels by 1 min, and increased again at 5 min by ~ 20 nmol. The PI content decreased maximally by ~ 6 nmol

(40%) at 5 s and returned to control levels by 30 s with both alkalosis and ATP, but was unchanged thereafter. Mass-balance analysis of net changes in DAG and PI pools suggests that additional sources, possibly PC, must also contribute to the DAG increase. ATP or alkalosis also increased the hydrolysis of PC. The labelling of phosphocholine was increased (~ 60%) at as early as 5 s and remained elevated at subsequent time points, whereas labelling of choline was higher only with ATP at 50 s and later, suggesting activation of phospholipase C by both agonists, and of phospholipase D by only ATP. Our studies demonstrate that ATP and alkalosis stimulate rapid hydrolysis of inositol and choline phospholipids to increase the DAG mass in type II cells, and that phospholipase C-stimulated PC hydrolysis is the major pathway for DAG formation.

INTRODUCTION

The lung surfactant phosphatidylcholine (PC) is stored in the lamellar bodies of alveolar type II cells before its secretion into the alveolar lumen. Among various mechanisms suggested for regulation of lung surfactant secretion, protein kinase C (PKC) activation has been postulated to contribute significantly [1–6]. Our previous studies also showed a role for PKC in the stimulation of surfactant secretion by alkalosis, caused by lowering of CO₂ concentration in the ventilation or incubation gas mixture [1,2].

1,2-Diacylglycerol (DAG) is the intracellular messenger for activation of PKC in a wide variety of cells. In type II cells, hydrolysis of (poly)phosphoinositides is stimulated by ATP [4,6,7], and the concomitant increase in labelling of DAG and inositol phosphates suggests activation of phospholipase C (PLC). DAG, an activator of PKC, can also be formed by PLC-dependent hydrolysis of PC or phosphatidylethanolamine (PE), or by phospholipase D (PLD)-dependent hydrolysis of PC and subsequent hydrolysis of phosphatidic acid thus formed by phosphatidate phosphatase [8,9]. During our investigations on regulation of lung surfactant secretion, we observed that the alkalosis- and ATP-stimulated surfactant secretion from type II cells is inhibited by the PKC inhibitor staurosporine. Since this observation suggested that alkalosis and ATP activated similar pathways of stimulation of surfactant secretion, we measured alkalosis- and ATP-stimulated changes in DAG content and (poly)phosphoinositide and PC hydrolysis in type II cells. Our results demonstrate that each of these agents induces rapid hydrolysis of PC and phosphatidylinositol (PI), and a biphasic increase in the DAG content of type II cells. A mass-balance

analysis of changes in PI and DAG also supports that multiple metabolic sources (and pathways) are utilized for DAG increase with both alkalosis and ATP. Some of this work was previously presented in abstract form [2,10].

MATERIALS AND METHODS

Materials

[methyl-³H]Choline, [γ-³²P]ATP, dipalmitoyl phosphatidyl-[methyl-¹⁴C]choline, D-*myo*-[2-³H]inositol, D-*myo*-[2-³H]inositol 1-phosphate (potassium salt), D-*myo*-[2-³H]inositol 1,4-bisphosphate (potassium salt) and D-*myo*-[³H]inositol 1,4,5-trisphosphate (potassium salt) were obtained from Amersham Corp., Arlington Heights, IL, U.S.A. Elastase was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. DAG kinase and staurosporine were obtained from Calbiochem, La Jolla, CA, U.S.A. Cardiolipin was purchased from Avanti Polar Lipids, Pelham, AL, U.S.A. Plastic tissue-culture dishes were obtained from Costar, Cambridge, MA, U.S.A. Rat serum IgG, DNAase I, DAG, phosphatidic acid, PI and other standard chemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Tissue-culture media were obtained from Gibco BRL, Grand Island, NY, U.S.A. Bacteriological plates (Falcon 1029) were purchased from Beckton and Dickinson, Franklin Lakes, NJ, U.S.A. Whatman LK-5 t.l.c. plates were obtained from Baxter Health Care, Edison, NJ, U.S.A.

Isolation of type II cells

Type II epithelial cells were isolated after elastase digestion of lungs from pathogen-free male Sprague–Dawley rats (180–200 g)

Abbreviations used: DAG, 1,2-diacylglycerol; IP₁, inositol 1-phosphate; IP₂, inositol bisphosphate; IP₃, inositol trisphosphate; MEM, minimum essential medium; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP₂, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C.

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[11]. In brief, lungs were cleared of blood by perfusion with solution I (2.6 mM phosphate buffer, pH 7.4, 10 mM Hepes, pH 7.4, 155 mM NaCl, 10 mM glucose, 1.74 mM CaCl_2 , 5.2 mM KCl, 1.3 mM MgSO_4 , 10 $\mu\text{g}/\text{ml}$ streptomycin and 100 $\mu\text{g}/\text{ml}$ penicillin), lavaged eight times with solution II (Ca^{2+} - and Mg^{2+} -free solution I containing 0.15 mM EGTA) and then three times with solution I. The lungs were then endotracheally treated for 3×10 min with elastase (3 units/ml), minced with a tissue chopper, and the minced tissue was suspended in solution I containing 0.2 mg/ml DNAase I. The minced tissue was shaken vigorously at 37 °C in a shaking water bath for 2 min to free the cells, which were sequentially filtered through 160 μm -, 37 μm - and 15 μm -mesh nylon cloth, and sedimented by centrifugation for 10 min at 300 g . The pelleted cells were suspended in minimum essential medium (MEM) and plated on bacteriological plates coated with 3 mg of IgG. After incubation for 1 h, the free cells were collected by 'panning' and pelleted by centrifugation for 10 min at 300 g . The pelleted cells were suspended in MEM containing 10% (v/v) fetal-bovine serum, plated on plastic tissue-culture dishes, and cultured for 20–22 h in 5% CO_2 in air. At the end of this culture period, > 90% of the attached cells were type II, as determined by phosphine 3R fluorescence, and > 95% of these were viable, as determined by exclusion of vital dye erythrosin B.

PC secretion

Type II cells (0.8×10^6) in 1.5 ml of MEM were incubated with 0.3 $\mu\text{Ci}/\text{ml}$ [*methyl*- ^3H]choline (sp. radioactivity 80 Ci/mmol) for 20–22 h in order to label cellular PC. The cells on the dishes were then washed six times with MEM and three times with the incubation buffer, containing 117.6 mM NaCl, 25 mM NaHCO_3 , 4.7 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 1.3 mM CaCl_2 , 30 mM Hepes, pH 7.4, and 10 mM glucose. The buffer was equilibrated with 5% CO_2 in air to give a final pH of 7.4. Fresh incubation buffer (2 ml) was added to the culture dishes, and the cells were equilibrated for 30 min in 5% CO_2 . At the end of this period, two plates were removed for determination of total radioactivity in the lipids of media and the cells in order to calculate PC release during the equilibration period (zero time). Other culture dishes were incubated for the next 2 h without or with addition of 1 mM ATP. In some experiments, some of the culture dishes were transferred to 0% CO_2 (alkalosis, due to increased $[\text{HCO}_3^-]/\text{CO}_2$ ratio) and the incubations continued for 2 h. At the end of each incubation, the medium was aspirated and centrifuged at 300 g for 10 min to remove any detached cells, which were pooled with those on the plate. Lipids from the medium and cells were extracted [12] after addition of dipalmitoylglycerophospho[^{14}C]choline as a recovery standard and egg PC as a carrier lipid. Percentage secretion was calculated from corrected radioactivities by the equation:

$$\text{Secretion (\%)} = \frac{\text{d.p.m. in media lipids} \times 100}{(\text{d.p.m. in media lipids} + \text{d.p.m. in cell lipids})}$$

PC hydrolysis

Cells (1×10^6) in 1.5 ml of MEM were labelled with 1.5 μCi of [*methyl*- ^3H]choline for 20–22 h during overnight culture, washed with MEM (five times) and then equilibrated for 30 min in fresh MEM without serum and in 5% CO_2 in air. Thereafter, some of the tissue-culture dishes were placed in 0% CO_2 in air, whereas either 15 μl of stock solution of ATP (final concn. 1 mM) or

0.9% NaCl was added to others and incubations were continued for indicated periods of time up to 10 min. Incubations were staggered so as to time the incubation period accurately. However, it may be noted that 5 s elapsed between termination of incubation and addition of chloroform/methanol (1:2, v/v) to the plates, which was not added to the incubation period. At the end of each incubation, the cells and the medium were extracted for lipids [12], and the radioactivity in the lipid extract was measured. The aqueous phases from lipid extracts from cells and the medium were pooled, freeze-dried, and reconstituted in 10 μl of aq. 100 mM choline and 50 mM phosphocholine. A portion (3 μl) was then used for separation of choline and phosphocholine by t.l.c. on silica-gel G plates that were developed in 0.5% NaCl/methanol/ NH_3 (50:50:1, by vol.) [13]. Choline and phosphocholine on t.l.c. plates were revealed after exposure to I_2 vapour, scraped off, and counted for ^3H label in a scintillation counter. The radioactivity in each metabolite was then expressed as a percentage of total ^3H label in the cells and the medium.

Formation of inositol phosphates

Cells ($5 \times 10^6/2.5$ ml) were incubated with 7 $\mu\text{Ci}/\text{ml}$ [^3H]inositol (sp. radioactivity 10–20 Ci/mmol) for 20–22 h to label cellular PI, and then washed five times with MEM and three times with the incubation buffer. The cells were then equilibrated for 30 min in 2 ml of incubation buffer containing 20 mM LiCl and 10 mM sodium orthovanadate and 5% CO_2 in air. At the end of this equilibration period, some culture dishes were incubated for various periods of time in the absence or presence of 1 mM ATP, or after transfer to 0% CO_2 (alkalosis). Individual incubations were terminated by rapid aspiration of the medium and addition of 2 ml of ice-cold methanol. After 15 min, the cells were scraped off and the steps repeated, and the pooled samples were extracted for lipids [12]. The upper aqueous phase from the lipid-extraction procedure was used to measure water-soluble inositol phosphates, and the lower organic phase was used to determine DAG and PI. Because of the brief periods of incubation, the incubations were staggered so that only one culture dish was handled at any time. The incubation medium did not contain LiCl and orthovanadate in experiments where only PI and DAG were measured. In some experiments, 100 nM staurosporine (10 μl was added from a stock solution of 200 μM in dimethylformamide, which had been diluted with incubation buffer without bicarbonate to 10 μM) was present during the equilibration and subsequent incubation periods.

Inositol phosphates were separated by anion-exchange chromatography on AG1-X8 (formate form) after neutralization of a sample of the upper phase with KOH [14]. Briefly, after loading of the sample, free inositol was eluted with water (15 ml), glycerophosphoinositol with 5 mM sodium tetraborate/60 mM sodium formate (15 ml), inositol 1-phosphate (IP_1) with 200 mM ammonium formate/100 mM formic acid (15 ml), inositol bisphosphate (IP_2) with 400 mM ammonium formate/100 mM formic acid (15 ml), and inositol trisphosphate (IP_3) with 1 M ammonium formate/100 mM formic acid (15 ml). Radioactive standards for IP_1 , IP_2 and IP_3 were run through the column to verify separation of individual inositol phosphates by using the protocol described above. The recovery of radioactive standards in their respective solution system exceeded 75%.

Determination of PI and DAG mass

PI levels in lipid extracts were measured by charring densitometry [15]. Lipid samples were spotted on 20 cm $\times$ 20 cm boric acid-impregnated LK-5 t.l.c. plates [16], the plates were dried under

vacuum at 25 °C for 20 min, and the lipids were separated by t.l.c. in chloroform/ethanol/ammonia/water (30:50:9:6, by vol.). The plates were then dried under vacuum at 100 °C for 20 min, sprayed with 10% CuSO₄ in 8% H₃PO₄, heated at 120 °C for 7 min, and charred for 12 min at 170 °C on a heating block. The lipid quantities were then measured by scanning densitometry of charred lipids at 440 nm. Various amounts of authentic PI were also run on each plate, and a standard curve was constructed.

DAG levels in lipid extracts of type II cells were measured after phosphorylation to phosphatidic acid [17]. Samples of lipid extract were dried under a stream of N₂ and solubilized as mixed micelles in 20 µl of a solution containing 7.5% octyl β-D-glucoside, 5 mM cardiolipin and 1 mM diethylenetriaminepenta-acetic acid (DETAPAC) by sonication in a bath sonicator for 15 s. For each 10 µl of DAG kinase (20 m-units), 50 µl of 2× reaction buffer (100 mM imidazole/HCl, pH 6.6, 100 mM NaCl, 25 mM MgCl₂, 2 mM EGTA), 10 µl of 20 mM dithio-threitol and 10 µl of 10 µM [γ-³²P]ATP (sp. radioactivity 4 Ci/mmol) in buffer (100 mM imidazole and 1 mM DETAPAC, pH 6.6) were added. An 80 µl portion of this solution was then added to each of the 20 µl sonicated lipid extracts and incubated at 25 °C for 30 min. The reaction was terminated by addition of 1 ml of chloroform/methanol (1:2, v/v) and processed for lipid extraction [12]. The lower phase was washed once with 2 ml of 1% HClO₄, and dried under N₂. The lipids were dissolved in 50 µl of chloroform/methanol (20:1, v/v), and the phospholipids separated by t.l.c. in chloroform/methanol/acetic acid (13:3:1, by vol.). Lipid, co-migrating with authentic phosphatidic acid, was scraped off and counted for radioactivity in a liquid-scintillation counter. In preliminary studies with known amounts of DAG, we observed quantitative conversion of DAG into [³²P]phosphatidic acid (*r* = 0.99). Therefore the DAG mass (nmol) in the sample was calculated from the c.p.m. in [³²P]phosphatidic acid divided by c.p.m./nmol of ATP. Recovery of DAG during lipid extraction was determined by adding known amounts of 1,2-dipalmitoylglycerol (100–700 pmol) to cells in chloroform/methanol (1:2, v/v). The recovery of added DAG was > 88% at each of these concentrations.

Phospholipid P was measured by the method of Marinetti [18] as described previously [19]. P was assumed to comprise 4% of the total mass of phospholipids. Results were first evaluated for differences by one-way or two-way analysis of variance and then analysed statistically by using the paired (within a cell preparation) or unpaired Student's *t* test. The statistical significance was accepted at *P* < 0.05.

RESULTS

Both ATP and alkalosis stimulated the secretion of surfactant PC from type II cells. During a 2 h incubation, PC secretion in cells exposed to alkalosis increased by ~200%, and in those exposed to 1 mM ATP increased by almost 400% (Table 1) (*n* = 4, *P* < 0.05). In the presence of 100 nM staurosporine, the increase in PC secretion was only 20% with alkalosis (*P* > 0.05 versus control) and 150% with ATP (*P* < 0.05 versus control or ATP without inhibitor). A partial inhibition of ATP-stimulated secretion is compatible with activation of multiple pathways of signal transduction by ATP [3].

Previous reports have suggested that ATP- and alkalosis-stimulated secretion is mediated via activation of PKC [1,4], and ATP stimulation of type II cells is associated with increased labelling of DAG [4,7]. In our hands alkalosis and ATP also increased the formation of DAG in type II cells labelled with

[³H]arachidonic acid (results not shown). Since the increase in the content, rather than the labelling, of DAG determines the activation of PKC, we investigated changes in DAG content as a function of time under basal conditions or in cells stimulated with ATP or alkalosis. The basal level of DAG was 50 ± 1.1 nmol/mg of phospholipid (*n* = 8), which did not change up to 30 min of incubation (Figure 1). However, both alkalosis and ATP rapidly increased the DAG content of cells. The pattern of change was similar with both ATP and alkalosis. Cells treated with either 1 mM ATP or alkalosis showed a time-dependent biphasic increase in DAG content. Compared with controls, the DAG content rapidly increased by 100% at 5 s, remained around the same level up to 20 s, and then gradually declined to near basal level by 1 min. It was again higher by ~30–40% at 5, 10 and 30 min of incubation with each stimulus

Table 1 Staurosporine inhibition of alkalosis- and ATP-induced PC secretion

Results are means ± S.E.M. of experiments conducted with the numbers of cell preparations indicated in parentheses. Where indicated, staurosporine (100 nM) was present during the equilibration and subsequent 2 h incubation period. **P* < 0.05 versus corresponding control; †*P* < 0.05 versus without inhibitor.

Staurosporine ...	PC secretion (% in 2 h)	
	—	+
Control	0.66 ± 0.06 (17)	0.64 ± 0.10 (15)
Alkalosis	2.07 ± 0.31* (12)	0.81 ± 0.15† (10)
ATP (1 mM)	3.20 ± 0.49* (9)	1.69 ± 0.19† (9)

Figure 1 DAG content of type II cells as a function of time in response to ATP and alkalosis

Type II cells were equilibrated for 30 min in Krebs–Ringer bicarbonate buffer, pH 7.4, containing 10 mM glucose and 30 mM Hepes, pH 7.4, and in 5% CO₂ in air (zero time) before the addition of 1 mM ATP or transfer to 0% CO₂ in air (alkalosis). Incubations were terminated at indicated times by rapid aspiration of the media and addition of chloroform/methanol (1:2, v/v). Results are means ± S.E.M. of experiments on 3–4 cell preparations and are expressed as percentages of control at zero time. Control DAG content at zero time was 50 ± 1.1 nmol/mg of phospholipid (*n* = 8). **P* < 0.05 for ATP or alkalosis versus control.

Figure 2 Time courses of [^3H]choline and [^3H]phosphocholine formation with alkalosis and ATP

Type II cells were labelled with [$^3\text{methyl-}^3\text{H}$]choline during overnight culture, washed, equilibrated for 30 min in 5% CO_2 in air and exposed to agonists as given for Figure 1. Radioactivity in choline and phosphocholine was measured in the pooled aqueous fractions obtained during extraction of cell and medium lipids for each time point. Choline and phosphocholine accounted for 0.6 ± 0.1 and $5.5 \pm 0.9\%$ of total ^3H label. Results are expressed as percentages of label at zero time and are means $\pm$ S.E.M. of experiments with four separate cell preparations: * $P < 0.05$ for ATP or alkalosis versus control.

($P < 0.05$). Although we did not measure DAG content at time points intermediate between 10 and 30 min, we assume that the second increase in DAG content was of a sustained nature, as against a transient increase between 5 and 20 s.

Agonist activation of cells, including type II cells, can induce PC hydrolysis and generate DAG by PLC- or PLD-catalysed reactions [8,20–22]. Next, we investigated the effect of alkalosis and ATP on PC hydrolysis and monitored the labelling of choline and phosphocholine in cells labelled with [$^3\text{methyl-}^3\text{H}$]choline during 20 h in culture. Under these conditions $> 95\%$ of lipid label is in cell PC. In control cells, 0.6 ± 0.1 and $5.5 \pm 0.9\%$ ($n = 3$, mean $\pm$ S.E.M.) of the total ^3H was present in choline and phosphocholine fractions respectively. The relative labelling of choline and phosphocholine is lower than that reported by Dubrovin and Brown [20], who cultured type II cells on fibronectin-coated plates and conducted experiments with cells in suspension. Both ATP and alkalosis increased the labelling

Figure 3 Effect of alkalosis and ATP on inositol phosphates as a function of time

Cells labelled with [^3H]inositol during overnight culture were washed, equilibrated for 30 min in 5% CO_2 in air, and then incubated for indicated periods of time as given for Figure 1. Results are expressed as percentages of control, and are means $\pm$ S.E.M. of experiments with five cell preparations. Radioactivity in control cells at 15 s was 46.5 ± 15.9 in IP_1 , 10.7 ± 2.5 in IP_2 and 7.6 ± 2.1 in IP_3 (d.p.m./ μg of phospholipid). * $P < 0.05$ versus control. Symbols: $\circ$, control; $\bullet$, ATP; ∇ , alkalosis.

of phosphocholine by $\sim 60\%$ within 5 s of treatment, which remained elevated for up to 10 min (Figure 2). Assuming that PC is labelled to equilibrium under these conditions, as previously shown by others [20], we can calculate that approx. 3% of cell PC is hydrolysed by PLC to yield phosphocholine and DAG within 5 s of treatment. Since PC comprises 67% of type II cell phospholipids [19], the PLC-catalysed PC hydrolysis represents

Figure 4 PI content of type II cells in response to ATP and alkalosis

Isolated type II cells were treated as given for Figure 1. Results are expressed as percentages of control level at zero time and are means $\pm$ S.E.M. of experiments with four or five cell preparations. PI content at zero time was 14 ± 0.8 ($n = 7$) nmol/mg of phospholipid. * $P < 0.05$ for ATP or alkalosis versus control.

Table 2 Secretagogue-stimulated changes in PI and DAG content of Type II cells

Cells were washed, equilibrated for 30 min in 5% CO_2 in air, and then incubated for 5 s after addition of 1 mM ATP or transfer to 0% CO_2 in air (alkalosis). Results are means $\pm$ S.E.M. of 5 experiments: * $P < 0.05$ versus controls.

	Lipid content (nmol/mg of total phospholipids)		
	Control	Alkalosis	ATP (1 mM)
PI	14.1 ± 0.5	$7.5 \pm 0.3^*$	$8.2 \pm 0.5^*$
DAG	46.8 ± 0.7	$87.2 \pm 1.8^*$	$96.8 \pm 4.9^*$
Δ PI		7.2 ± 0.3	5.9 ± 0.7
Δ DAG		40.4 ± 1.3	50.0 ± 4.5

2% of total phospholipids and can increase DAG by 50%. The pattern of changes in choline labelling was somewhat different. First, only ATP stimulated choline formation, and second, this increase was apparent only at 50 s or later time points (Figure 2). Thus choline can be formed either via sequential hydrolysis of PC by PLC and of phosphocholine by a phosphatase, or from direct hydrolysis of PC by PLD. PC hydrolysis via the latter pathway appears to be a minor component under our experimental conditions. Taken together, our results show that ATP and alkalosis stimulate PLC-mediated hydrolysis of PC, which may be the major pathway to increase DAG in type II cells.

Another potential pathway for DAG increase is PI metabolism. In fact, the agonist-stimulated increase in DAG is generally believed to be solely derived from PLC-catalysed hydrolysis of (poly)phosphoinositides in a number of cells, including type II cells [7,23–25]. In our hands also, ATP increased the formation of all three inositol phosphates in type II cells prelabelled with

[^3H]inositol (Figure 3). [^3H]IP $_3$ increased from 7.6 ± 2.1 to 38.4 ± 7.5 c.p.m./ μg of phospholipid within 15 s, remained elevated up to 5 min, and then returned to near normal by 30 min (Figure 3). The changes at 15, 30, 60 and 300 s represented increases of 450, 280, 280 and 370%, respectively, above basal values. ATP also increased the formation of IP $_1$ and IP $_2$ at all the time points except at 30 min. The increases in IP $_1$ and IP $_2$ were around 150% and 500% respectively at every time point except at 30 min. In contrast, alkalosis failed to enhance the formation of any of inositol phosphates at all time points investigated (Figure 3). Thus, our studies suggest that ATP, but not alkalosis, stimulates (poly)polyphosphoinositide-specific phospholipase C in type II cells.

We also investigated the effect of ATP and alkalosis on the PI content of type II cells. The basal level of PI was 14 ± 0.8 nmol/mg of phospholipid ($n = 7$). A time course of changes in the mass of PI with ATP and alkalosis is shown in Figure 4. Our earliest observation was at 5 s, at which time the PI content showed a maximal decrease of $\sim 40\%$ with either ATP or alkalosis. Thereafter, the PI content returned towards the control level by 30 s and was unchanged thereafter up to 30 min. Since the PI decrease parallels the DAG increase, a portion of DAG can come from PI hydrolysis.

Determination of PI and DAG contents enabled us to carry out mass-balance analysis of changes in these two lipids in secretagogue-stimulated type II cells (Table 2). After 5 s of treatment, compared with corresponding controls, the DAG content was higher by 50 nmol/mg of total phospholipid with ATP, and by 40 nmol/mg of total phospholipid with alkalosis. In comparison, the PI content decreased by about 6–7 nmol/mg of phospholipid with both ATP and alkalosis. Thus only about 10% of the change in DAG could be accounted for by PI hydrolysis.

PKC plays a regulatory role in DAG formation by inducing PC hydrolysis [20], or inhibition of PLC-catalysed phosphatidylinositol 4,5-bisphosphate (PIP $_2$) hydrolysis [26]. To investigate whether PKC played any role in DAG increase, we evaluated the effect of staurosporine on the early increase in DAG content with alkalosis and ATP. We anticipated that staurosporine inhibition of PKC would alter the secretagogue-induced increase in the DAG content. In four separate experiments, the DAG content of control cells was 48 ± 6 and 43 ± 8 nmol/mg of phospholipid respectively in the absence or presence of 100 nM staurosporine which was added at the start of equilibration period of 30 min. Under these conditions, a 30 s treatment with alkalosis and ATP increased the DAG content to 70 ± 6 ($P < 0.05$) and 66 ± 10 ($P < 0.05$) nmol/mg respectively in the absence of staurosporine. In cells pretreated with staurosporine, the DAG content increased to 63 ± 7 and 60 ± 4 ($P < 0.05$ versus control) nmol/mg with alkalosis and ATP respectively. Thus staurosporine-mediated inhibition of PKC did not affect the early increase in the DAG content of type II cells with either agonist.

DISCUSSION

This study demonstrates that two secretagogues of lung surfactant, ATP and alkalosis, induce time-dependent rapid changes in DAG and PI contents of type II cells. Although the decrease in PI was transient and returned to normal by 30 s, the increase in DAG was transient during the early phase and was sustained during the later phase. In addition, the two agonists also induced rapid hydrolysis of PC that was primarily catalysed by PLC. Thus these agonists mobilize multiple precursors (and pathways) for DAG increase in type II cells.

Previous investigations employing metabolic labelling protocols provided useful information about metabolic pathways induced by an agonist. Thus ATP is suggested to induce PLC-mediated hydrolysis of (poly)phosphoinositides and to increase [^3H]IP₃ and [^3H]DAG formation in type II cells [4,6,7]. However, labelling protocols determine only relative changes in DAG [7,20], since all of the metabolic lipid pools may not be labelled to the same specific radioactivity, and therefore may not accurately represent their mass distribution [27]. Therefore it is important to establish whether any of these agents induce changes in DAG content.

The DAG and PI contents in type II cells have not been determined previously by other investigators. It is noteworthy that the DAG pool of type II cells is much larger than the PI pool (4% and 1.2% respectively of total phospholipids). This is strikingly different from tracheal tissue preparations, showing 4% PI and 0.3% DAG [15]. This difference is possibly reflected in the observation that the stimulus-induced change in PI is greater than that in DAG of tracheal preparations [15]. In type II cells, their relative contents and the changes in PI and DAG contents showed a reverse order.

Our findings on the initial phases of changes in DAG and PI contents suggest a direct relationship between these two lipids (Figures 1 and 4), and are in agreement with previous reports employing metabolic labelling-kinetics protocols [7,8]. However, our mass-balance analysis indicates that only a small amount (~10%) of DAG increase may arise from PI, and that a large proportion of the early increase is derived from other sources (Table 2). This analysis also suggests that the hydrolysis of all the cell PI, the precursor for (poly)phosphoinositides, cannot fully account for the total increase in DAG. Our calculations do not take into account the metabolic utilization of DAG via DAG kinase or DAG lipase, as previously demonstrated in human platelets [28,29]. Activation of these two enzymes for the removal of DAG will only underestimate the DAG increase. That the DAG levels were higher at later time points, and the PI content was unchanged (Figures 1 and 4), also implies utilization of other phospholipids for the DAG increase. Other investigators also drew similar conclusions from mass-balance analysis of stimulus-induced changes in IP₃ and DAG in Swiss 3T3 cells [23].

Our studies on PC hydrolysis support activation of PLC by alkalosis, and of both PLC and PLD by ATP, to form phosphocholine and choline. The labelling profiles for choline and phosphocholine permit calculation of the relative contributions of PLC- and PLD-catalysed hydrolysis of PC. Assuming that PC is labelled to equilibrium, any changes in the labelling of phosphocholine and choline suggest quantitative hydrolysis of PC via PLC- and PLD-catalysed reactions. Our assumption is also supported by the fact that the ratio of label recovered in choline and phosphocholine (0.6% and 5.5% respectively) in control cells is similar to the ratio of their pool sizes previously reported in type II cells or lungs [30,31]. We suggest that PC hydrolysis via PLD is unlikely to contribute significantly to the DAG pool. This is based on the observations that the labelling of choline was unaltered with alkalosis, and was increased only after 50 s with ATP. Also, the increased labelling of choline with ATP represented hydrolysis of about 0.4% of cell PC. This is similar to ATP-stimulated PLD-mediated phosphatidylethanol formation in type II cells labelled with acetate [22]. In comparison, a rapid increase in the labelling of phosphocholine demonstrates that each agonist stimulates PLC-catalysed hydrolysis of PC, which can contribute almost half of the DAG increase. Although various investigators have suggested that both PLC- and PLD-catalysed hydrolysis of PC contributes to the DAG increase [8,21,24,32–34], our results demonstrate

that only PLC-dependent breakdown of cellular PC in type II cells contributes significantly. On the basis of the rapid labelling of phosphocholine, we further suggest that the plasma-membrane PC is the preferred substrate. Previous studies have shown that agonists (ATP) increase PLC-mediated hydrolysis of PC in rat liver plasma membrane [24].

The underlying mechanisms for the biphasic increase in DAG are not known, but may be related to the presence of multiple substrates. Other investigators also observed a biphasic increase in DAG content in bombesin-stimulated Swiss 3T3 fibroblasts and suggested that DAG may be derived from PC [23]. It has been suggested that PIP₂ hydrolysis may provide the first increase and serve to initiate DAG generation from an alternative source, probably PC or PE [8,9,23,24,32,35]. This is supported by the observations that the molecular-species profile of DAG in thrombin-stimulated fibroblasts resembles both PI and PE profiles at early time points and the PC profile at later time points [36]. However, it is unlikely that PIP₂ hydrolysis is required for the second increase in DAG in type II cells. First, alkalosis did not increase IP₃ formation, but caused a biphasic increase in DAG (Figures 1 and 3), and second, there was no secondary increase in phosphocholine or choline label (Figure 2) to suggest PC hydrolysis at another intracellular site. A likely explanation for the second DAG peak is that other phospholipids, possibly PE, are mobilized.

In contrast with their similar effects on DAG and PI contents, the effects of ATP and alkalosis on the formation of inositol phosphates and choline (Figure 3) were different. In agreement with previous investigators [6,7,20,22], we also observed that ATP increased the PLC-specific hydrolysis of (poly)phosphoinositides and PLC- and PLD-specific hydrolysis of PC. However, the mechanism by which PI breakdown is increased with alkalosis is unknown. Since we did not observe accumulation of inositol phosphates, we suggest that alkalosis activates PI-specific PLD, which would increase PI hydrolysis to form phosphatidic acid and inositol, and has been previously characterized in human neutrophils [37]. Since PKC activation stimulates PLD-catalysed hydrolysis of phospholipids [20,22], and ATP, but not alkalosis, increased choline formation (Figure 2), there may also be differences in the type of PKC activated by each of these agonists.

PKC modulates phospholipid hydrolysis in a dual manner. In type II cells, phorbol 12-myristate 13-acetate (a direct activator of PKC) activates PC hydrolysis [20], suggesting a positive co-operativity between PKC activation and DAG generation. In mesangial cells, angiotensin- and vasopressin-induced and PLC-activated hydrolysis of (poly)phosphoinositides can be attenuated by short-term activation of PKC [38,39], suggesting a feedback inhibition of PLC (and DAG generation) by PKC. Since DAG content was unchanged in staurosporine-treated cells, we suggest that PKC does not contribute significantly to the DAG increase by mechanisms discussed above. However, staurosporine inhibition of surfactant secretion supports a role for PKC that may be activated after the DAG increase.

In conclusion, we have demonstrated that ATP and alkalosis stimulate the hydrolysis of PC and (poly)phosphoinositides, and increase the DAG content and surfactant secretion in type II cells. We suggest that PLC-catalysed PC hydrolysis contributes significantly to the increase in DAG content, whereas a smaller proportion may be derived from (poly)phosphoinositide hydrolysis. The enzymic pathways catalysing hydrolysis of (poly)phosphoinositides appear to be different in ATP and alkalosis. The physiological role for the biphasic increase and the relative importance of the two increases in DAG in the regulation of surfactant secretion remain to be determined.

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REFERENCES

- 1 Chander, A. (1989) *Am. J. Physiol.* **257** (Lung Cell Mol. Physiol. 1), L354–L360
- 2 Chander, A. and Williams, S. J. (1990) *Am. Rev. Respir. Dis.* **141**, A635 (abstr.)
- 3 Griesse, M., Gobran, L. I. and Rooney, S. A. (1991) *Am. J. Physiol.* **260** (Lung Cell Mol. Physiol. 4), L140–L147
- 4 Rice, W. R., Dorn, C. C. and Singleton, F. M. (1990) *Biochem. J.* **266**, 407–413
- 5 Sano, K., Voelker, D. R. and Mason, R. J. (1985) *J. Biol. Chem.* **260**, 12725–12729
- 6 Warburton, D., Buckley, S. and Cosico, L. (1989) *J. Appl. Physiol.* **66**, 901–905
- 7 Griesse, M., Gobran, L. I. and Rooney, S. A. (1991) *Am. J. Physiol.* **260** (Lung Cell Mol. Physiol. 4), L586–L593
- 8 Billah, M. M. and Anthes, J. C. (1990) *Biochem. J.* **269**, 281–291
- 9 Kiss, Z. and Anderson, W. B. (1990) *J. Biol. Chem.* **265**, 7345–7350
- 10 Sen, N. and Chander, A. (1992) *FASEB J.* **6**, A1269 (abstr.)
- 11 Dobbs, L. G., Gonzalez, R. and Williams, M. C. (1986) *Am. Rev. Respir. Dis.* **134**, 141–145
- 12 Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- 13 Yavin, E. (1976) *J. Biol. Chem.* **251**, 1392–1397
- 14 Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. and Irvine, R. F. (1983) *Biochem. J.* **212**, 473–482
- 15 Baron, C. B., Cunningham, M., Strauss, J. F., III and Coburn, R. F. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6899–6903
- 16 Fine, J. B. and Sprecher, H. (1982) *J. Lipid Res.* **23**, 660–663
- 17 Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E. and Bell, R. M. (1986) *J. Biol. Chem.* **261**, 8597–8600
- 18 Marinetti, G. V. (1962) *J. Lipid Res.* **3**, 1–20
- 19 Chander, A., Fisher, A. B. and Strauss, J. F., III (1982) *Biochem. J.* **208**, 651–658
- 20 Dubrovin, L. C. and Brown, L. A. S. (1992) *Am. J. Physiol.* **263** (Lung Cell Mol. Physiol. 6), L42–L50
- 21 Exton, J. H. (1990) *J. Biol. Chem.* **265**, 1823–1826
- 22 Rooney, S. A. and Gobran, L. I. (1993) *Am. J. Physiol.* **264** (Lung Cell. Mol. Physiol. 8), L133–L140
- 23 Cook, S. J., Palmer, S., Plevin, R. and Wakelam, M. J. O. (1990) *Biochem. J.* **265**, 617–620
- 24 Irving, H. R. and Exton, J. H. (1987) *J. Biol. Chem.* **262**, 3440–3443
- 25 Takashi, M. and Williams, J. A. (1989) *J. Biol. Chem.* **264**, 14729–14734
- 26 Berridge, M. J., Cobbold, P. H. and Cuthbertson, K. S. R. (1988) *Philos. Trans. R. Soc. London* **B320**, 325–343
- 27 Mehadevappa, V. G. and Holub, B. J. (1984) *Can. J. Biochem.* **62**, 827–830
- 28 Bishop, W. R. and Bell, R. M. (1986) *J. Biol. Chem.* **261**, 12513–12519
- 29 Bishop, W. R., Ganong, B. R. and Bell, R. M. (1986) *J. Biol. Chem.* **261**, 6993–7000
- 30 Post, M., Batenburg, J. J., Smith, B. T. and van Golde, L. M. G. (1984) *Biochim. Biophys. Acta* **795**, 552–557
- 31 Yost, R. W., Chander, A., Dodia, C. and Fisher, A. B. (1986) *J. Appl. Physiol.* **61**, 2040–2044
- 32 Bocckino, S. B., Blackmore, P. F. and Exton, J. H. (1985) *J. Biol. Chem.* **260**, 14201–14207
- 33 Kennerly, D. A. (1987) *J. Biol. Chem.* **262**, 16305–16313
- 34 Lacal, J. C., Moscat, J. and Aaronson, S. A. (1987) *Nature (London)* **330**, 269–272
- 35 Daniel, L. W., Waite, M. and Wykle, R. L. (1986) *J. Biol. Chem.* **261**, 9128–9132
- 36 Pessin, M. S. and Raben, D. M. (1989) *J. Biol. Chem.* **264**, 8729–8738
- 37 Balsinde, J., Diez, E., Fernandez, B. and Mollineda, F. (1989) *Eur. J. Biochem.* **186**, 717–724
- 38 Pfeilschifter, J. (1986) *FEBS Lett.* **203**, 262–266
- 39 Troyer, D. A., Gonzalez, O. F., Douglas, J. G. and Kreisberg, J. I. (1988) *Biochem. J.* **251**, 907–912

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