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P_2-purinoceptors regulate surfactant secretion from rat isolated alveolar Type II cells

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1 Rat isolated alveolar Type II cells were utilized to examine the effect of purine and pyrimidine analogues on secretion of pulmonary surfactant.

2 ATP potently stimulated [^{3}H]-phosphatidylcholine ([^{3}H]-PC) secretion in a time- and dose-dependent manner. The effect of ATP was noted by one hour of exposure and persisted for three hours. The EC_{50} (concentration producing 1/2 the maximal response) for ATP-induced [^{3}H]-PC secretion was 100 nM.

3 ADP was also a potent secretagogue for surfactant secretion, but AMP and adenosine had no significant effect on surfactant secretion at concentrations $< 250\,\mu M$. The EC_{50} for ADP-induced [^{3}H]-PC secretion was 250 nM.

4 Other purine and pyrimidine nucleotides (ITP, GTP, CTP, TTP) were examined for their effect on [^{3}H]-PC secretion. All purine and pyrimidine triphosphates examined significantly augmented [^{3}H]-PC secretion, but were much less potent than ATP. The EC_{50}s were ITP = $10\,\mu M$; GTP = $100\,\mu M$; CTP = $250\,\mu M$; TTP = $100\,\mu M$.

5 Neither 8-phenyltheophylline ($10\,\mu M$, a P_1-purinoceptor antagonist), propranolol ($100\,\mu M$, a β-adrenoceptor antagonist), nor indomethacin ($10\,\mu M$, a prostaglandin synthetase inhibitor) inhibited ATP-induced [^{3}H]-PC secretion from isolated Type II cells.

6 These data provide evidence for regulation of surfactant secretion from alveolar Type II cells by a P_2-purinoceptor.

Introduction

Alveolar Type II epithelial cells synthesize and secrete lamellar bodies which form pulmonary surfactant on the alveolar surface. Lamellar bodies are secreted from alveolar Type II cells in response to a variety of secretagogues which include β-adrenoceptor agonists (Dobbs & Mason, 1979; Mettler *et al.*, 1981; Brown & Longmore, 1981), forskolin (Rice *et al.*, 1985), cholera toxin (Mescher *et al.*, 1983), cytochalasins (Rice *et al.*, 1984), prostaglandins (Gilfillan & Rooney, 1985), phorbol esters (Dobbs & Mason, 1978), and ionophore A23187 (Mason *et al.*, 1977; Marino & Rooney, 1980). The precise mechanism by which each of these agents augments release of surfactant is not known. Release of lamellar bodies induced by β-adrenoceptor agonists, forskolin, and cholera toxin is associated with elevated adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels which in turn activate cyclic AMP-dependent protein kinase (Rice *et al.*, 1985). However, the substrates for this protein kinase remain to be elucidated. A role of calcium in regulating surfactant release has also been suggested on the basis of ionophore A23187-induced surfactant secretion (Mason *et al.*, 1977; Marino & Rooney, 1980) and activation of calcium, phospholipid-dependent protein kinase in association with surfactant secretion (Sano *et al.*, 1985). Ionophore A23187 is known to augment cytosolic calcium levels in many cells, raising the possibility that elevated levels of cytosolic calcium might mediate surfactant release, although an endogenous agent which mediates changes in Type II cell cytosolic calcium levels has not been identified.

Many visceral organs of vertebrates including the lung are innervated by neurones, termed purinergic nerves by Burnstock (1972, 1980, 1981), that utilize adenosine 5′-triphosphate (ATP) as a neurotransmitter. Burnstock (1980) has proposed two types of purinoceptors based on rank order of agonist potency. For P_1-purinoceptors, the rank order of agonist potency is adenosine > AMP > ADP > ATP, while this rank order of agonist potency is reversed for P_2-purinoceptors (Burnstock, 1980). Exogenous ATP increases insulin secretion from the isolated perfused

pancreas of the rat (Chapal & Loubatières-Mariani, 1981a, b) and increases amylase secretion from mouse parotid acinar cells (Gallacher, 1982). The purinoceptor regulating secretion in each case appears to be a P_2-type since ATP was the most effective secretagogue inducing insulin and amylase secretion and cyclic AMP, AMP and adenosine displayed only very weak activity (about 100 fold less acitve) (Loubatières-Mariani *et al.*, 1979) or no activity (Gallacher, 1982).

Previous workers utilizing rat lung slices demonstrated ATP-induced disaturated phosphatidylcholine secretion at supramaximal concentrations of ATP (1 mM) (Gilfillan *et al.*, 1983). This ATP effect was hypothesized to be due to cyclic AMP which was produced from ATP, since cyclic AMP is known to mediate surfactant secretion as noted above (Rice *et al.*, 1985). An alternative exlanation is that Type II cells in rat lung slices contain receptors for ATP which regulate surfactant secretion. This hypothesis was tested in the present work utilizing isolated alveolar Type II cells from rat lung.

Methods

Animals

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

Isolation and culture of Type II cells

Type II cells were isolated from rat lungs as previously described (Dobbs & Mason, 1979; Mettler *et al.*, 1981; Brown & Longmore, 1981). 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 the lungs perfused via the pulmonary artery with buffer A (NaCl 125 mM, KCl 5 mM, Na_2HPO_2 2.5 mM, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) 17 mM, glucose 1 mg ml^{-1}, gentamycin sulphate 10 μg ml^{-1}, and penicillin 20 u ml^{-1}, pH 7.4) containing 3% bovine serum albumin (w/v). Bovine serum albumin diluted in buffer A (90 ml, 1 mg ml^{-1}) and fluorocarbon (3 ml, 3M Co., St Paul, MN) were sonicated and 12 ml were instilled into the lungs of each rat via the cannulated trachea to facilitate removal of macrophages by centrifugation. After removal of this solution, trypsin (3 mg ml^{-1}) was instilled into the lungs which were incubated for 20 min at 37°C. Digestion was terminated by addition of 10 ml (1 mg ml^{-1}) soybean trypsin inhibitor. Tissue was minced and filtered through Nitex gauze and washed in buffer A. Type II cells were then purified by discontinuous albumin density gradient centrifugation as previously described (Brown & Longmore, 1981). Isolated cells were resuspended in Dulbecco's Modified Eagle's medium (GIBCO, Grand Island, NY) containing 10% foetal calf serum and utilized for experiments following 18 h in culture at 37°C in 5% CO_2. This procedure routinely yielded 10×10^6 cells per rat. For release experiments, cells were diluted in plating medium to $0.5–1.0 \times 10^6$ cells per ml. Cell suspension (1.0 ml) was placed in each well of a 24 well tissue culture plate (Falcon 3047) which had been precoated with collagen (Collaborative Research, Lexington, MA) and prelabelled by addition of 1 μCi of [^{3}H]-choline (sp. act., 80.0 Ci mmol^{-1}). The plating efficiency at 20 h was generally 40%. Non-adherent cells were removed from the wells by washing before the assay. Cultures contained 90–95% viable Type II cells as determined by trypan blue exclusion, fluorescent staining with phospine 3R, and electron microscopy of sample plates.

Secretion of phosphatidylcholine

Secretion of phosphatidylcholine (PC) by cultured Type II cells was assessed by a modification of previously published methods by determining [^{3}H]-PC release (Rice *et al.*, 1984). After 18 h in culture, cells were washed three times using Dulbecco's Modified Eagle's medium containing 40 mM HEPES and 3.0 mg ml^{-1} bovine serum albumin, pH = 7.4, 37°C. Cells were then re-equilibrated for 30 min and at the end of this time, agents were added and [^{3}H]-PC released was determined at the times noted. Medium was aspirated and the cells washed with 0.5 ml of fresh medium. The two samples of medium were then combined and centrifuged at 250 *g* for 5 min to pellet loose cells. Supernatant was removed and the lipid extracted according to Folch *et al.* (1957) with addition of 1.0 mg of egg PC as a carrier.

Cells remaining in the wells were extracted with 2×1.0 ml of methanol and the lipid fraction was obtained as for the medium. Samples containing lipid were dried overnight and radioactivity determined with a β-scintillation counter after addition of 5.0 ml of Scintiverse II to each sample. Dipalmitoyl [^{14}C]-phosphatidylcholine was used as an internal standard to follow recoveries of [^{3}H]-PC which were generally 95 ± 1%. The amount of [^{3}H]-PC secretion was calculated as the percentage of total [^{3}H]-PC present in the medium relative to the amount present in cells and medium (i.e., c.p.m. in medium/(c.p.m. in medium + c.p.m. in cells)). The amount of [^{3}H]-PC released following the 30 min pre-incubation was subtracted from all samples. Preliminary experiments, in which labelled lipids were separated by thin layer chromatography before scintillation counting, indicated 90–95% of the radioactivity was present in the [^{3}H]-PC fraction, as previously found by other work-

ers (Mettler *et al.*, 1981). Manipulations involved in isolating [^{3}H]-PC would introduce errors in excess of that represented by contaminating lipids (Mettler *et al.*, 1981).

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]propanesulphonic acid, pH = 7.0, and 0.1 ml of 10 mM NADH, freshly prepared. The reaction was initiated by adding 0.1 ml of sodium pyruvate and the enzyme activity determined by following the decrease in absorbance at 340 nm (Rice *et al.*, 1984). Total activity was determined by treating sample plates with 0.5% Triton X-100. None of the agents utilized for these experiments resulted in a statistically significant release of lactate dehydrogenase above control levels which were 1–2% of total cellular lactate dehydrogenase released after a 3 h incubation.

Statistical analyses

Kruskal-Wallis one-way analysis of variance by ranks (Kruskal & Wallis, 1952) or Friedman (1937) two-way analysis of variance by ranks for non-parametric data were utilized as appropriate. Analysis of variance was performed on an Apple IIe microcomputer using ANOVA II (Human System Dynamics, Northridge, CA). $P < 0.05$ was considered statistically significant. All experiments were performed in triplicate, except those depicted in Figure 1 which were single determinations.

Materials

[Methyl-^{3}H]-choline chloride and dipalmitoyl [^{14}C]-phosphatidylcholine were purchased from New England Nuclear (Boston, MA). Chloroform, methanol, and Scintiverse II were from Fisher Chemical Co. (Cincinnati, OH). Disodium ethylenediaminetetraacetate (EDTA), trypsin, indomethacin, soybean trypsin inhibitor, 8-phenyltheophylline, (±)-propranolol HCl, ATP, adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), adenosine, guanosine 5′-triphosphate (GTP), inosine 5′-triphosphate (ITP), thymidine 5′-triphosphate (TTP) and cytidine 5′-triphosphate (CTP) were all obtained from Sigma Chemical Co. (St Louis, MO). Egg phosphatidylcholine (PC) was obtained from Avanti Polar Lipids (Birmingham, AL). Bovine serum albumin was purchased from Miles Laboratories (Elkhart, IN).

Results

ATP caused a time-dependent secretion of [^{3}H]-PC, relative to the control (Figure 1). ATP-induced [^{3}H]-PC secretion was statistically different from the control value by one hour of incubation (the first time point examined). This statistically significant augmentation of [^{3}H]-PC secretion persisted for 3 h ($P < 0.05$).

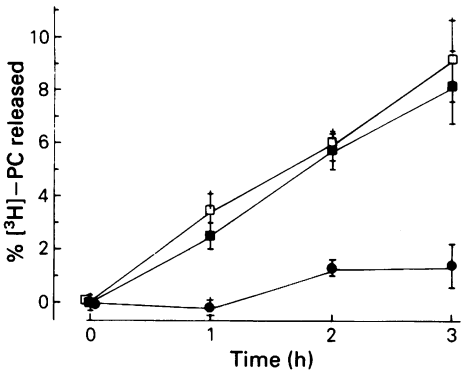

Figure 1 Time course for ATP-augmented [^{3}H]-phosphatidylcholine ([^{3}H]-PC) secretion. Release of [^{3}H]-PC was determined after exposure of isolated Type II cells to buffer (●), 1 μM ATP (■) or 10 μM ATP (□) for the indicated length of time. [^{3}H]-PC release is expressed as stated in Methods. Data shown represent the mean of six experiments and vertical lines indicate s.e.mean. ATP at 1 and 10 μM produced a significant ($P < 0.001$) augmentation of surfactant secretion, relative to the control, at 1, 2 and 3 h of exposure. Release induced by 1 μM ATP was not significantly different ($P > 0.05$) from release induced by 10 μM ATP.

To determine whether this effect was mediated by a purinoceptor, we performed a dose-response analysis for adenosine and adenosine derivatives (Figure 2). ATP was the most potent secretagogue examined. The EC_{50} for ATP-induced surfactant secretion was 100 nM. ADP was also an effective secretagogue with an EC_{50} of 250 nM. AMP and adenosine had no statistically significant effect on [^{3}H]-PC secretion at concentrations < 250 μM.

Effects of other purine and pyrimidine triphosphates on surfactant secretion were next examined. ATP was the most potent purine triphosphate (Figures 3 and 4). ITP produced significant stimulation of [^{3}H]-PC secretion with an EC_{50} of 10 μM while GTP was less effective with an EC_{50} of 100 μM. The pyrimidine triphosphates TTP and CTP were less potent secretagogues relative to ATP (Figure 4), with EC_{50}s of 100 μM and 250 μM, respectively.

Since stimulation of [^{3}H]-PC secretion by ATP appeared to be mediated by a P_2-purinoceptor on the basis of relative potency of agonists (ATP>ADP>>AMP = adenosine), we examined the effect of 8-phenyltheophylline (a P_1-purinoceptor antagonist) on sur-

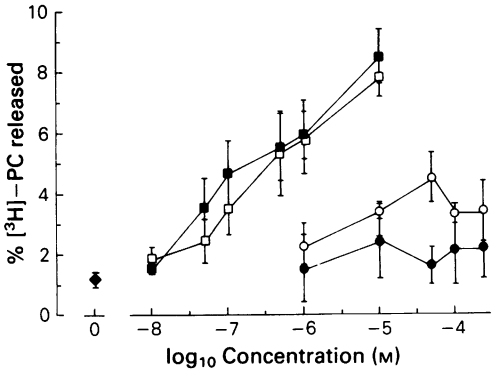

Figure 2 Effect of ATP, ADP, AMP and adenosine on [^{3}H]-phosphatidylcholine ([^{3}H]-PC) release. Release of [^{3}H]-PC was determined after a 3 h exposure of Type II cells to ATP (■), ADP (□), AMP (●), adenosine (○) or buffer (◆) at the indicated concentrations. Release of [^{3}H]-PC is expressed as stated in Methods. Data represent the mean ($n = 4$) and vertical lines show s.e.mean. ATP ($P < 0.001$) and ADP ($P = 0.007$) caused significant augmentation of [^{3}H]-PC release. AMP ($P > 0.25$) and adenosine ($P = 0.15$) did not significantly stimulate [^{3}H]-PC secretion at the concentrations tested.

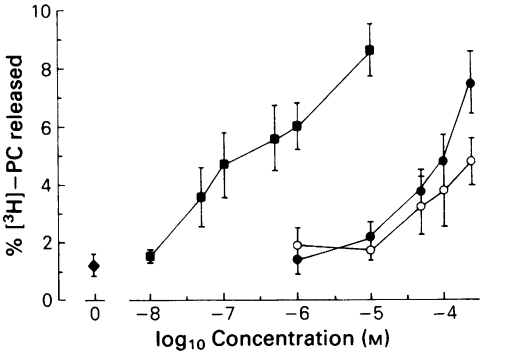

Figure 4 Effect of ATP and pyrimidine nucleotide triphosphates on [^{3}H]-PC release. Release of [^{3}H]-PC was determined after a 3 h exposure of Type II cells to ATP (■), cytidine 5′-triphosphate (CTP, ○), thymidine 5′-triphosphate (TTP, ●) or buffer (◆) at the indicated concentrations. Release of [^{3}H]-PC is expressed as stated in Methods. Data represent the mean ($n = 3$) and vertical lines show s.e.mean. ATP ($P < 0.001$), CTP ($P = 0.03$) and TTP ($P = 0.007$) all caused significant stimulation of [^{3}H]-PC release.

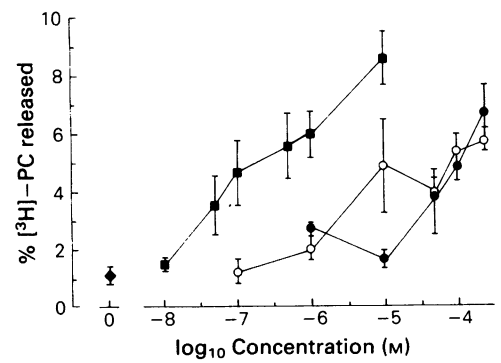

Figure 3 Effect of purine nucleotide triphosphates on [^{3}H]-phosphatidylcholine ([^{3}H]-PC) release. Release of [^{3}H]-PC was determined after a 3 h exposure of Type II cells to ATP (■), inosine 5′-triphosphate (ITP, ○), guanosine 5′-triphosphate (GTP, ●) or buffer (◆) at the indicated concentrations. Release of [^{3}H]-PC is expressed as stated in Methods. Data represent the mean ($n = 3$) and vertical lines show s.e.mean. ATP ($P < 0.001$), ITP ($P > 0.001$) and GTP ($P = 0.002$) all caused significant augmentation of [^{3}H]-PC release.

factant secretion induced by ATP. 8-Phenyltheophylline had no significant effect on surfactant secretion induced by 10 μM ATP (Table 1). Similarly, the β-adrenoceptor antagonist propranolol was without effect on ATP-induced [^{3}H]-PC secretion (Table 1), but did inhibit β-agonist-induced (terbutaline) [^{3}H]-PC secretion. The prostaglandin synthetase inhibitor indomethacin was also without effect on ATP-induced [^{3}H]-PC secretion (Table 1).

Discussion

Exogenous ATP is a potent stimulus for surfactant secretion from isolated alveolar Type II cells maintained in primary culture. Our findings are analagous to those of Loubatières-Mariani *et al.* (1979) who demonstrated a stimulatory effect of exogenous ATP on insulin secretion from the isolated, perfused pancreas and Gallacher (1982) who showed ATP-induced amylase secretion from mouse parotid acinar cells. Our data are also consistent with the findings of Gilfillan *et al.* (1983) demonstrating ATP-induced surfactant secretion from rat lung slices. Regulation of secretion from pancreatic β-cells, parotid acinar cells and lung alveolar Type II cells appears to be mediated by P_2-purinoceptors, rather than P_1-purinoceptors. In the present case, ATP and ADP were potent

Table 1 Effect of 8-phenyltheophylline, propranolol, and indomethacin on ATP- and terbutaline-induced [^{3}H]-phosphatidylcholine ([^{3}H]-PC) secretion

Agent	*%[^{3}H]-PC released*	n
Control	1.4 ± 0.3*	10
8-Phenyltheophylline	1.3 ± 0.1	5
Propranolol	0.6 ± 0.3	5
Indomethacin	1.1 ± 0.4	4
ATP	8.3 ± 1.0	5
ATP + 8-phenyltheophylline	8.4 ± 0.8	4
ATP + propranolol	7.1 ± 1.0	5
ATP + indomethacin	8.5 ± 0.8	4
Terbutaline	5.2 ± 0.8	4
Terbutaline + 8-phenyltheophylline	4.3 ± 0.6	6
Terbutaline + propranolol	1.5 ± 0.5	5

Release of [^{3}H]-PC was determined after a 3 h exposure of Type II cells to ATP (10 μM), 8-phenyltheophylline (10 μM), propranolol (100 μM), indomethacin (10 μM), terbutaline (10 μM) or combinations of these agents. Data represent the mean ± s.e.mean for the number of experiments shown (*n*). *8-Phenyltheophylline, propranolol and indomethacin had no significant effect on control or ATP-induced [^{3}H]-PC secretion. ATP ($P < 0.001$) and terbutaline ($P = 0.005$) significantly augmented [^{3}H]-PC release. Propranolol ($P < 0.05$) significantly inhibited terbutaline-induced [^{3}H]-PC secretion.

secretagogues relative to adenosine and AMP which were ineffective at stimulating [^{3}H]-PC release. 8-Phenyltheophylline (a potent antagonist of P_1-purinoceptors (Smellie *et al.*, 1979; Griffith *et al.*, 1981)) and propranolol (a β-adrenoceptor antagonist) were without effect on ATP-induced surfactant secretion.

The present experiments are consistent with those conducted previously when comparing the efficacy of various purine and pyrimidine nucleotides. In the rat pancreas, ATP had the strongest insulin secretory effect with GTP and ITP being less effective in that order (Loubatières-Mariani *et al.*, 1979). Pyrimidine nucleotides CTP and UTP were without effect in the rat pancreas, similar to the present study where CTP and TTP were 1000 times less potent than ATP. For the P_2-purinoceptor mediating endothelium-dependent relaxation of the pig aorta, ATP had the greatest effect with CTP, TTP, ITP and UTP being 100 times less potent (Martin *et al.*, 1985), similar to our findings.

The EC_{50} we observed for ATP-induced [^{3}H]-PC secretion (100 nM) is lower than the EC_{50} for ATP-induced calcium mobilization in isolated hepatocytes previously reported (800 nM) (Charest *et al.*, 1985), but of the same order of magnitude. Type II cells also appear to be more sensitive than other ATP-sensitive tissues like guinea-pig taenia coli ($EC_{50} = 1$ μM) (Burnstock *et al.*, 1983) and rat caecum ($EC_{50} = 10$ μM) (Mehta & Kulkarni, 1983).

The mechanism by which occupancy of Type II cell P_2-purinoceptors results in augmented surfactant secretion is unknown. ATP stimulates prostacyclin secretion from cultured porcine endothelial cell monolayers (Pearson *et al.*, 1983). Extracellular ADP and ATP increase release of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) in rabbit aorta, rabbit pulmonary artery and rat aorta (Boeynaems & Galand, 1983), and occupancy of P_2-purinoceptors in the pulmonary porcine vasculature results in increased release of PGI_2 (Hellewell & Pearson, 1984). Previous work has demonstrated that prostaglandins increase secretion of surfactant from isolated Type II cells maintained in tissue culture (Gilfillan & Rooney, 1985). While it is possible ATP causes increased prostacyclin concentrations in Type II cells, the effect of prostaglandins on surfactant secretion from Type II cells previously described (Gilfillan & Rooney, 1985) was much smaller than the effect of ATP on Type II cell surfactant secretion described here and the prostaglandin synthetase inhibitor, indomethacin, was without effect on ATP-induced [^{3}H]-PC release in the present study.

Exogenous application of ATP also results in a selective increase in potassium permeability in intestinal smooth muscle cells (Fedan *et al.*, 1984). Apamin, a potent neurotoxin from bee venom, blocks this ATP-induced increase in potassium conductance and is known to interact with calcium-dependent potassium channels (Banks *et al.*, 1979). The phorbol ester TPA which is a potent secretagogue for surfactant secretion from Type II cells (Dobbs & Mason, 1978) and acts via activation of calcium, phospholipid-dependent protein kinase (Sano *et al.*, 1985), is also known to increase potassium conductance in 3T3 cells (Sussman

et al., 1985). In rat isolated hepatocytes, occupation of P_2-purinoceptors results in formation of inositol triphosphate and mobilization of intracellular calcium, presumably by augmenting phosphatidylinositol turnover (Charest *et al.*, 1985). Whether occupation of P_2-purinoceptors of Type II cells also results in formation of inositol triphosphate and mobilization of intracellular calcium with subsequent changes in potassium conductance or activation of calcium, phospholipid-dependent protein kinase remains to be demonstrated.

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