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P_{2Y}-purinoceptor regulation of surfactant secretion from rat isolated alveolar type II cells is associated with mobilization of intracellular calcium

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1 The effect of methylene, thio, and imido substituted analogues of adenosine 5'-triphosphate (ATP) on surfactant phospholipid secretion and calcium mobilization in rat isolated alveolar Type II cells was studied.

2 ATP was the most potent secretagogue of adenine nucleotides studied. The rank order of agonist potency for [^{3}H]-phosphatidylcholine secretion was ATP > adenosine 5'-O-(3-thiotriphosphate) (γS-ATP) > β, γ-imido adenosine 5'-triphosphate (AMPPNP) > β, γ-methylene adenosine 5'-triphosphate (β, γ-CH_2-ATP) > α, β-methylene adenosine 5'-triphosphate (α, β-CH_2-ATP). The respective EC_{50}s were 10^{-6} M, 2×10^{-6} M, 2×10^{-5} M, 5×10^{-5} M, and $> 2.5 \times 10^{-4}$ M.

3 Exogenous ATP also induced a rapid mobilization of intracellular calcium monitored by changes in Fura 2 fluorescence. The rank order of agonist potency for calcium mobilization was similar to the rank order of agonist potency for surfactant secretion: ATP = γS-ATP > AMPPNP > α, β-CH_2-ATP.

4 There was no effect of EGTA on ATP-induced calcium mobilization, consistent with the hypothesis that exogenous ATP induces release of calcium from intracellular stores.

5 These data are consistent with a P_{2Y}-purinoceptor regulating surfactant secretion from isolated Type II cells via mobilization of intracellular calcium, since: (a) non-hydrolyzed analogues of ATP are potent secretagogues, (b) β, γ-CH_2-ATP was a more potent secretagogue than α, β-CH_2-ATP and (c) the rank orders of agonist potency for calcium mobilization and phospholipid secretion were the same.

Introduction

Type II alveolar epithelial cells synthesize and secrete pulmonary surfactant in response to a variety of secretagogues (Hollingsworth & Gilfillan, 1984). Adenosine 5'-triphosphate (ATP) is one of the most potent exogenous stimuli for surfactant secretion from isolated Type II cells through activation of purinoceptors (Rice & Singleton, 1986; Gilfillan & Rooney, 1987). However, the precise mechanism by which P_2-purinoceptors augment surfactant secretion is not known.

In isolated hepatocytes of the rat, occupation of P_2-purinoceptors results in formation of inositol triphosphate and mobilization of intracellular calcium, presumably by augmenting phosphatidylinositol polyphosphate turnover (Charest *et al.*, 1985). Extracellular ATP also induces calcium mobilization in Ehrlich ascites tumour cells and endothelial cells (Dubyak & DeYoung, 1985; Lückhoff & Busse, 1986).

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A role of calcium in regulating surfactant release from Type II cells has been suggested on the basis of ionophore A23187-induced surfactant secretion (Mason *et al.*, 1977, Marino & Rooney, 1980) and activation of calcium phospholipid-dependent kinase in association with surfactant secretion (Sano *et al.*, 1985). Ionophore A23187 is known to augment cytsolic calcium levels in many cell types (Fleisch & Haisch, 1982), 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 prior to this time. Since exogenous ATP is a potent stimulus for phospholipid secretion from isolated alveolar Type II cells and exogenous ATP is known to mobilize cytosolic calcium in other systems, we tested the hypothesis that P_2-purinoceptors of alveolar Type II cells regulate surfactant secretion in association with mobilization of intracellular calcium.

In addition, we sought to define the subtype of P_2-purinoceptor responsible for regulation of surfactant secretion. Several subtypes of P_2-purinoceptors have been proposed based on rank order of analogue affinity for the receptor (Burnstock & Kennedy, 1985; Gordon, 1986). T subtype receptors are found on platelets and thrombocytes and bind methylene-substituted adenosine 5'-triphosphates with lower affinity than ATP (Gordon, 1986). P_{2X}-purinoceptors bind such analogues with higher affinity than ATP while P_{2Y}-purinoceptors which are found on pancreatic cells and parotid acinar cells also bind methylene-substituted derivatives with lower affinity than ATP (Burnstock & Kennedy, 1985; Gordon, 1986). Z subtype receptors are found on lymphocytes and mast cells and also bind methylene-substituted derivatives with lower affinity than ATP. By utilizing methylene-substituted derivatives of ATP in the present work, we sought to test whether P_{2T}, P_{2X}, P_{2Y} or P_{2Z} subtype purinoceptors are responsible for regulation of surfactant secretion in isolated Type II cells.

Methods

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 recently described by Dobbs *et al.* (1986). Rats were maintained under sterile guard hooded cages and allowed food and water *ad libidum* before the experiment. Rats were then anaesthetized with sodium pentobarbitone and lungs perfused via the pulmonary artery with buffer A (NaCl 125 mM, KCl 5 mM, Na_2HPO_4 2.5 mM, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) 17 mM, glucose 1 mg ml^{-1}, gentamycin 10 μg ml^{-1}, penicillin 20 u ml^{-1}, pH 7.4) containing 3% bovine serum albumin (w/v). Lungs were then lavaged as previously described (Rice & Singleton, 1986), and incubated with elastase solution (40 ml, 0.4 units ml^{-1} in buffer A) for 20 min at 37°C (Dobbs *et al.*, 1986). 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 and incubated on 100 mm bacteriological plastic Petri dishes that had been precoated with IgG 500 μg ml^{-1} for 3 h at room temperature. Following a 1 h incubation at 37°C in 5% CO_2/air, the plates were 'panned' as described (Dobbs *et al.*, 1986) and the unattached cells removed and collected by centrifugation. Isolated cells were resuspended in Dulbecco's Modified Eagle's medium (GIBCO, Grand Island, NY, U.S.A.) containing 10% foetal calf serum and used for experiments following 18 h in culture at 37°C in 5% CO_2. This procedure routinely yielded 40×10^6 cells per 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 that had been precoated with collagen (Collaborative Research, Lexington, MA, U.S.A.) and prelabelled by addition of 1 μCi of [^{3}H]-choline, (specific activity, 80.0 Ci $mmol^{-1}$). The plating efficiency at 18 h was generally 40–50%. Non-adherent cells were removed from the wells by washing before the assay. Cultures contained 89 ± 2% viable Type II cells as determined by fluorescence staining with phosphine 3R.

Secretion of phosphatidylcholine

Secretion of phosphatidylcholine (PC) by cultured Type II cells was determined as previously described (Rice & Singleton, 1986). Briefly, cells incubated overnight were washed 3 times with Dulbecco's Modified Eagle's medium containing 40 mM HEPES and 3 mg ml^{-1} bovine serum albumin, pH 7.4, 37°C (buffer B). Cells were allowed to equilibrate 30 min and at the end of this time, agents were added and [^{3}H]-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 9,000 *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 (Folch *et al.*, 1957).

Cells remaining in the wells were extracted with 1 ml methanol × 2 and fractions 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 (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 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 determined by following the decrease in absorbance at 340 nm. Total activity was deter-

mined by treating sample plates with 0.1% Triton X-100. None of the agents used 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 calcium

Intracellular calcium levels were determined by modification of a previously published method using the fluorescent calcium binding dye, Fura 2 (Dubyak, 1986). After cells had plated down overnight, they were scraped from plates with a rubber policeman, spun briefly in a microfuge (15 s × 9,000 *g*), and combined in 1 ml of buffer B. Fura 2 (5×10^{-6} M) was then added to the cell suspension. Cells were incubated for 10 min at 37°C, centrifuged at 9,000 *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 C (145 mM sodium chloride, 5 mM potassium chloride, 20 mM HEPES, 10 mM glucose, 1 mM Na_2HPO_4, pH 7.4) and incubated at 37°C. Fluorescence was monitored by use of an excitation wavelength of 339 nm and an emission wavelength of 500 nm. Each sample was standardized following cell lysis with Triton X-100 (0.1%). The fluorescence maximum was determined in the presence of 1 mM calcium and the fluorescence minimum in the presence of alkaline 2 mM EGTA (Dubyak, 1986). Cell autofluorescence was determined with unloaded cells and the quantity of extracellular dye present was determined in the presence and absence of extracellular EGTA (1 mM). With this protocol, cells contained $1-2 \times 10^{-5}$ M Fura 2 and leakage of dye was $< 5\%\ h^{-1}$.

Statistical analyses

Kruskall-Wallis one-way analysis of variance by ranks (Kruskall & Wallis, 1952) or Friedman two-way analysis of variance by ranks (Friedman, 1937) for non-parametric data were 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.

Materials

Methyl [^{3}H]-choline chloride and dipalmitoyl [^{14}C]-phosphatidylcholine 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.). Ethylene glycol bis (β amino ethyl ether) N, N, N′ N′-tetraacetic acid (EGTA), and α, β-methyleneadenosine 5′-triphosphate (α, β-CH_2-ATP) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Adenosine 5′-0-(-3-thiotriphosphate) (γS-ATP), β,γ-imidoadenosine 5′-triphosphate (AMPPNP), β, γ-methyleneadenosine 5′-triphosphate (β,γ-CH_2-ATP) were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Egg phosphatidylcholine (PC) was obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Elastase was obtained from Cooper Biomedicals (Malvern, PA, U.S.A.).

Results

ATP caused a concentration-dependent augmentation of [^{3}H]-PC secretion and was the most potent ATP analogue examined (Figures 1 and 2). Methylene derivatives of ATP were less potent analogues (Figure 1). The EC_{50} for ATP-induced [^{3}H]-PC secretion was 10^{-6} M, while the EC_{50}s for the methylene derivatives were 5×10^{-5} M for β,γ-CH_2-ATP and $> 2.5 \times 10^{-4}$ M for α,β-CH_2-ATP. Compounds containing other substituents in place of the anhydride oxygen linking the β and γ phosphorous atoms of ATP were more potent secretagogues than methylene derivatives (Figure 2). The EC_{50} for γS-ATP-stimulated [^{3}H]-PC secretion was 2×10^{-6} M while the corresponding value for AMPPNP-stimulated [^{3}H]-PC secretion was 2×10^{-5} M.

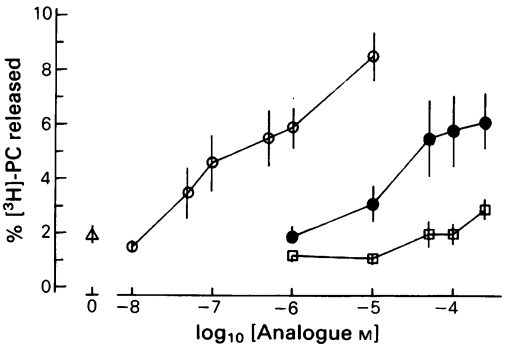

Figure 1 The effect of ATP, α, β-methyleneadenosine 5′-triphosphate (α, β-CH_2-ATP) and β, γ-methyleneadenosine 5′-triphosphate (β, γ-CH_2-ATP) on [^{3}H]-phosphatidylcholine ([^{3}H]-PC) release. Release of [^{3}H]-PC was determined after a 3 h exposure of Type II cells to ATP (○), α, β-CH_2-ATP (□), β,γ-CH_2-ATP (●), or buffer (△) at the indicated concentrations. Release of [^{3}H]-PC is expressed as stated in Methods. Data represent the mean ($n = 4$ experiments, 3 replicates in each) and vertical lines show s.e.mean. ATP, α, β-CH_2-ATP and β, γ-CH_2-ATP all caused significant augmentation of [^{3}H]-PC release.

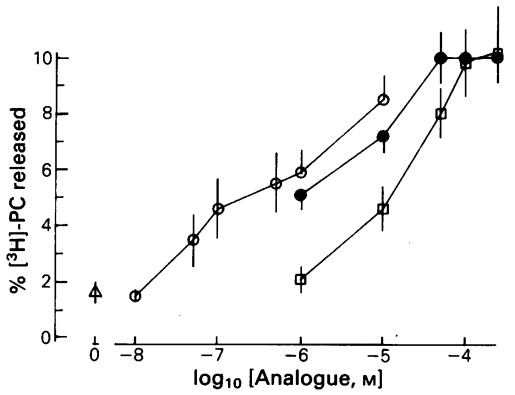

Figure 2 Effect of ATP, adenosine 5'-0-(3-thiotriphosphate) (γS-ATP), β, γ-imidoadenosine 5'-triphosphate (AMPPNP) on [^{3}H]-phosphatidylcholine ([^{3}H]-PC) release. Release of [^{3}H]-PC was determined after a 3 h exposure of Type II cells to ATP (○), γS-ATP (●), AMPPNP (□), or buffer (△) at the indicated concentrations. Release of [^{3}H]-PC is expressed as stated in Methods. Data represent the mean ($n = 4$ experiments, 3 replicates in each) and vertical lines show s.e.mean. ATP, γS-ATP, and AMPPNP all caused significant stimulation of [^{3}H]-PC release.

In order to define the mechanism by which P_2-purinoceptors of isolated Type II cells regulate [^{3}H]-PC secretion, we next determined intracellular calcium levels during stimulation with ATP and ATP analogues utilizing the calcium binding dye, Fura 2 (Figure 3). ATP caused a rapid increase in intracellular calcium levels determined by a marked increase in Fura 2 fluorescence (Figure 3). This effect of ATP was related to mobilization of intracellular calcium and not to the influx of extracellular calcium, since the peak cytosolic calcium level was unaffected by extracellular EGTA (Figure 3).

We next examined the effect of ATP analogues on mobilization of intracellular calcium (Figure 4 and Table 1). The rank order of agonist potency for calcium mobilization was similar to the rank order of agonist potency for induction of [^{3}H]-PC secretion. ATP and γS-ATP were the most potent secretagogues mobilizing intracellular calcium, followed by AMP-PNP. α, β-CH_2-ATP had no significant effect on cytosolic calcium concentrations at the concentration used for this set of experiments (1×10^{-5} M).

We also examined other agents which augment surfactant secretion from alveolar Type II cells and found the β-adrenoceptor agonist, terbutaline (1×10^{-5} M) and 12-0-tetradecanoyl phorbol 13-acetate (1×10^{-7} M) were without effect on intracellular calcium concentrations (not shown).

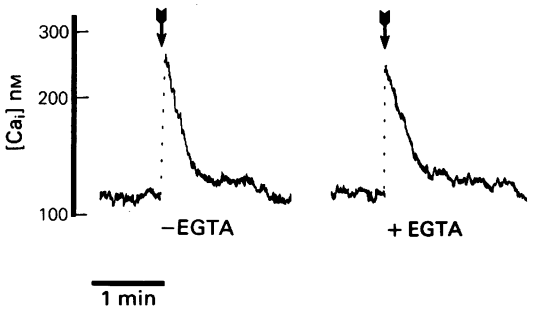

Figure 3 Effect of ATP on intracellular calcium levels in isolated Type II cells. Type II cells were preloaded with Fura 2 as described in Methods. ATP (1×10^{-5} M) was then added at the arrow in the presence or absence of 1×10^{-3} M EGTA. Extracellular calcium concentration was 1.5×10^{-4} M in the absence of EGTA. Intracellular calcium levels were determined as noted in Methods. The results presented are representative of 10 separate experiments.

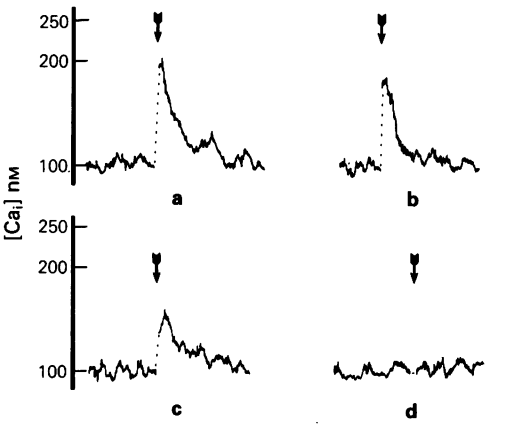

Figure 4 Mobilization of intracellular calcium by ATP and ATP analogues. Isolated alveolar Type II cells were preloaded with Fura 2 as noted in Methods and then exposed to 1×10^{-5} M ATP (a), 1×10^{-5} M adenosine 5'-0-(3-thiotriphosphate) (b), 1×10^{-5} M β,γ-imidoadenosine 5'-triphosphate (c), or 1×10^{-5} M α, β-methyleneadenosine 5'-triphosphate (d) at the times denoted by the arrows. The results shown are representative of 4 separate experiments.

Table 1 Effect of ATP and ATP analogues on cytosolic calcium levels

Analogue	*Peak Ca_i* (nM)
0	114 ± 5*
ATP	184 ± 4
γS–ATP	186 ± 9
AMPPNP	145 ± 4
α, β-CH_2-ATP	122 ± 6

* Cytosolic calcium concentrations were determined as noted in Methods. ATP or each analogue were present at 1×10^{-5} M. ATP, γS-ATP, and AMPPNP all significantly ($P < 0.05$) augmented cytosolic calcium concentrations at the levels used ($n = 4$ experiments). Under the conditions of this experiment, 1×10^{-5} M α, β-CH_2-ATP had no significant effect on the peak cytosolic calcium concentration. γS-ATP = adenosine 5'-0-(3-thiotriphosphate); AMPPNP = β, γ-imidoadenosine 5'-triphosphate; α, β-CH_2-ATP = α, β-methyleneadenosine 5'-triphosphate.

Discussion

Exogenous ATP is a potent secretagogue for surfactant secretion from isolated alveolar Type II cells maintained in primary culture. The data are consistent with a P_{2Y}-purinoceptor mediating this effect since β, γ-CH_2-ATP was a more potent secretagogue than α, β-CH_2-ATP. These results are consistent with those of Chapal & Loubatières-Mariani (1981) and Gallacher (1982) who demonstrated stimulatory effects of exogenous ATP on insulin secretion from the isolated, perfused pancreas and amylase secretion from mouse parotid acinar cells, respectively, which appear to be mediated by a P_{2Y}-purinoceptor. Hydrolysis of ATP is not necessary for induction of surfactant secretion, since non-hydrolyzed analogues of ATP and ADP are also effective secretagogues (Rice & Singleton, 1986).

The mechanism by which occupancy of Type II cell P_{2Y}-purinoceptors results in augmented surfactant secretion appears to involve mobilization of intracellular calcium. The rank order of agonist potency for mobilization of intracellular calcium was the same as the rank order of agonist potency for stimulation of [^{3}H]-PC secretion. The mechanism by which P_{2Y}-purinoceptor occupation leads to calcium mobilization is unknown for Type II cells, but may involve augmented phosphatidylinositol polyphosphate turnover with subsequent production of inositol triphosphate and diacylglycerol as demonstrated in isolated hepatocytes (Charest *et al.*, 1985), Ehrlich ascites tumour cells (Dubyak & DeYoung, 1985; Dubyak, 1986), and piglet aortic endothelial cells (Hallam & Pearson, 1986). Since diacylglycerol activates C-kinase, P_2-purinoceptor agonist binding may lead to C-kinase activation in isolated Type II cells. Previous data utilizing the phorbol ester, 12-0-tetradecanoyl phorbol 13-acetate (TPA), demonstrated activation of calcium phospholipid-dependent protein kinase of isolated alveolar Type II cells in association with surfactant secretion (Sano *et al.*, 1985). However, prior to the present work, an exogenous agent which could activate calcium, phospholipid-dependent protein kinase in isolated Type II cells has been lacking.

The phorbol ester TPA, and the β-adrenoceptor agonist, terbutaline, are both secretagogues for surfactant secretion (Rice *et al.*, 1985; Sano *et al.*, 1985) but failed to mobilize intracellular calcium. This is consistent with previous work demonstrating TPA-induced mobilization of C-kinase directly in association with surfactant secretion (Sano *et al.*, 1985) and terbutaline-induced activation of cyclic AMP-dependent protein kinase (Rice *et al.*, 1985). ATP is, therefore, the only agent currently available which acts extracellularly to induce mobilization of intracellular calcium in isolated alveolar Type II cells in association with surfactant secretion.

While ATP appears to be a potent stimulus for surfactant secretion *in vitro*, whether ATP plays a similar role *in vivo* and the source of ATP which could activate purinoceptors of the Type II cell *in vivo* is not clear. Surfactant secretion is enhanced by β-adrenoceptor stimulation and during hyperventilation (Oyarzún & Clements, 1977). Circulating catecholamine levels are elevated at birth and may account for the increased surfactant secretion observed at birth. However, ATP (which comprises 15% of the dry weight of adrenal granules) is co-secreted with adrenal catecholamines (Hillarp, 1958) and serum levels of ATP during the perinatal period are presently unknown. ATP is also released from vascular endothelial cells and smooth muscle cells in culture (Pearson & Gordon, 1979). Release of ATP by cultured cells appears selective, since ATP release is not accompanied by concomitant lactate dehydrogenase secretion (Pearson & Gordon, 1979). It is possible ATP may be generated *in vivo* by release from damaged cells, since the normal intracellular ATP concentration is 4–5 mM and Type II cells are sensitive to micromolar levels of ATP. Type II cells are known to respond to alveolar damage by secretion of surfactant prior to repopulating the alveolar lining. It is therefore possible that ATP may be the signal that controls Type II cell secretory mechanisms during such periods of stress. Experiments to test this hypothesis are currently in progress.

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