Calcium mobilization and response recovery following P₂-purinoceptor stimulation of rat isolated alveolar type II cells

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1 The effect of adenosine 5'-triphosphate (ATP) on surfactant phospholipid secretion, calcium mobilization, and the time course for recovery of the response system was studied in isolated alveolar Type II cells of the rat.

2 ATP (10 μ M) stimulated a biphasic intracellular Ca²⁺ transient monitored by changes in Fura-2 fluorescence, from a basal level of 126 ± 9 nm, to a rapid peak of 391 ± 1 nm, followed by a prolonged plateau 26 ± 4 nm above baseline (mean $\pm$ s.e.mean, $n = 26$).

3 ATP-stimulated surfactant phospholipid secretion and peak Ca²⁺ levels had similar EC₅₀s (1×10^{-6} M), and were unaffected by chelation of extracellular Ca²⁺. However, the prolonged plateau phase was abolished by chelation of extracellular Ca²⁺.

4 There was a 15 min refractory period before full recovery of the Ca²⁺-response to ATP. Recovery was dependent on extracellular Ca²⁺, was accelerated by removing extracellular agonist and was prolonged following stimulation with the poorly hydrolyzed ATP analogue, ATP- γ -S.

5 While the Type II cell was capable of multiple ATP-induced Ca²⁺ transients following recovery, no additional surfactant phospholipid was released with sequential stimulation.

6 These findings suggest initial exposure of Type II cells to ATP mobilizes intracellular Ca²⁺, stimulates phospholipid secretion and rapidly desensitizes the cell to further stimulation by ATP. Recovery of the ATP-induced Ca²⁺-response depends on presence of extracellular Ca²⁺ and removal of agonist.

Introduction

Exogenous adenosine 5'-triphosphate (ATP) interacts with P₂-purinoceptors to produce cellular responses in a variety of tissue types (Gordon, 1986). Exogenous ATP stimulates glycogenolysis in hepatocytes (Haussinger *et al.*, 1987), increases insulin secretion from the pancreas (Chapal & Loubatières-Mariani, 1981), augments amylase secretion from parotid acinar cells (Gallacher, 1982), and stimulates surfactant phospholipid secretion from alveolar Type II cells in primary culture (Rice & Singleton, 1986; Gilfillan & Rooney, 1988). P₂-purinoceptor stimulation generates second messengers from the breakdown of membrane phospholipids. In hepatocytes, stimulation of P₂-purinoceptors leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate and generation of myoinositol 1,4,5-triphosphate (IP₃) (Prpić *et al.*,

1982; Charest *et al.*, 1985). IP₃ acts on non-mitochondrial stores to release Ca²⁺ (Dubyak, 1986), and elevation of intracellular Ca²⁺ is associated with secretion in many cell types including pancreatic islet cells and alveolar Type II cells (Zawalich *et al.*, 1983; Dobbs *et al.*, 1986b). P₂-purinoceptor stimulation of Type II alveolar cells leads to an elevation of intracellular Ca²⁺ in association with surfactant phospholipid release (Rice & Singleton, 1987). Further work with Type II cells demonstrated rapid desensitization of the Ca²⁺-response upon restimulation with ATP (Dorn & Rice, 1988). Desensitization of agonist-induced response is common in biological systems, but the mechanism by which desensitization occurs in various cell types is diverse (Lefkowitz & Caron, 1986). Desensitization of the β -adrenoceptor-stimulated response in astrocytoma cells occurs by receptor sequestration (Toews *et al.*, 1984). In avian erythrocytes the response to β -

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adrenoceptor stimulation is modified by phosphorylation of the receptor and uncoupling of the GTP-dependent signalling protein (Sibley & Lefkowitz, 1985). In other cell types, desensitization is due to depletion of substrates necessary for the cellular response to agonist. Muscarinic stimulation of astrocytoma cells leads to depletion of an intracellular Ca^{2+} pool, desensitizing the cell until this calcium pool is refilled (McDonough *et al.*, 1988). Little is known about purinoceptor desensitization in any cell type, so the time course for response recovery in ATP-stimulated Type II cells and the factors which influence purinoceptor desensitization were investigated.

Methods

Isolation of Type II cells

Alveolar Type II cells were isolated from rat lungs by elastase digestion and selective plating on IgG as described previously (Dobbs *et al.*, 1986a). With this method, $30\text{--}40 \times 10^6$ Type II cells were routinely obtained per rat. Cell viability was approximately 90% as measured by trypan blue exclusion. Cell purity determined by phosphine 3R staining was $89 \pm 2\%$. Isolated cells were either used fresh or plated 18 h in Dulbecco's Modified Eagle medium (DMEM) with 10% foetal calf serum at 37°C in 5% CO_2 .

Measurement of intracellular calcium

Freshly isolated or cultured (18 h) cells were suspended in medium (DMEM + 40 mM HEPES + 3 mg ml⁻¹ bovine serum albumin with a final Ca^{2+} concentration of 1.4 mM) at a concentration of 15×10^6 cells ml⁻¹ and incubated with Fura-2AM (5 μM in dimethylsulphoxide, DMSO) at 37°C for 10 min. Cells were washed with fresh medium and incubated for an additional 10 min at 37°C . To terminate Fura-2AM loading, cells were washed three times with medium (4°C) and stored on ice until use. With this protocol, cells were uniformly labelled with Fura-2, without evidence of organellar sequestration as determined by fluorescence microscopy. Calcium sensitive fluorescence was measured with a fluorometer (excitation = 339 nm, emission = 500 nm), equipped with a thermostatted (37°C), magnetic stirrer. For each run, the concentration of intracellular Ca^{2+} in Fura-2 loaded cells was calculated as previously described (Rice & Singleton, 1987). For some time courses, cells were placed in a bath at 37°C shielded from light. In long time courses, signal drift from dye leakage was accounted for and amounted to <10% of incorporated dye.

Phospholipid secretion

Secretion of phosphatidylcholine (PC) from cultured Type II cells was determined by the methods described previously (Rice & Singleton, 1986). Briefly, freshly isolated cells were labelled by addition of 1 μCi [³H]-choline (sp. act. 80.0 Ci mm⁻¹) and plated overnight at 37°C in 5% CO_2 . Following 3 washes with fresh medium and a 30 min equilibration period, ATP was added to the plated cells. At 3 h, medium was aspirated and centrifuged to pellet loose cells. Lipid was extracted from the supernatant according to Folch *et al.* (1957). The remaining plated cells were extracted with methanol and the lipid fraction was obtained as for the medium. Lipid samples were dried overnight and radioactivity determined with a β scintillation counter after addition of Scintiverse II to the samples. The amount of [³H]-PC secreted was calculated as the percentage of [³H]-PC in the medium relative to the amount present in the cells and medium. The amount of [³H]-PC released during the 30 min preincubation was subtracted from all samples.

Lactate dehydrogenase release

Lactate dehydrogenase (LDH) activity was determined as a measure of cytotoxicity by methods described previously (Rice *et al.*, 1984). Release of LDH from ATP-treated, freshly isolated, Fura loaded cells and plated cells (18 h) was not significantly different from control values (0–4% of total LDH present in cells).

Statistical analysis

Analysis of variance was performed on an IBM-PC using commercially available statistical packages. $P < 0.05$ was considered statistically significant. All experiments were performed in triplicate unless noted.

Materials

Pathogen-free male Sprague-Dawley rats (200–250 g) were obtained from Charles River Laboratories (Wilmington, MA, U.S.A.). Elastase (porcine pancreas) was purchased from Cooper Biomedicals (Malvern, PA, U.S.A.), Fura-2-acetoxymethyl ester (Fura-2 AM) from Molecular Probes (Junction City, OR, U.S.A.), methyl [³H]-choline chloride and [¹⁴C]-dipalmitoyl phosphatidylcholine from New England Nuclear (Boston, MA, U.S.A.), egg phosphatidylcholine (PC) from Avanti Polar Lipids (Birmingham, AL, U.S.A.), adenosine 5'-O-(3-thiotriphosphate) (ATP- γ -S) from Boehringer-Mannheim Biochemicals (Indianapolis, IN, U.S.A.), and Dul-

Figure 1 Effect of ATP on intracellular calcium levels in freshly isolated Type II cells. Type II cells were loaded with Fura 2 as described in Methods. ATP (1×10^{-5} M) was added at the arrow in the absence (a), or presence (b) of EGTA (2×10^{-3} M). (c) ATP treatment of cells in presence of EGTA at time zero, then at the time noted, CaCl_2 (2×10^{-3} M) was added. Intracellular calcium levels were determined as described in methods. The results presented are representative of 26 separate experiments.

becco's Modified Eagle Medium (DMEM) from Gibco (New York, NY, U.S.A.). Chloroform, methanol and Scintiverse II were purchased from Fisher Chemical (Cincinnati, OH, U.S.A.). IgG (rat), DNase, adenosine 5'-triphosphate (ATP), 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES), ethylene glycol bis (β -aminoethylether)- N,N,N',N' -tetraacetic acid (EGTA) and bovine serum albumin were obtained from Sigma (St. Louis, MO, U.S.A.).

Results

Treatment of freshly isolated Type II cells with ATP ($10 \mu\text{M}$) resulted in a rapid peak of intracellular Ca^{2+} , followed by a prolonged plateau phase (Figure 1a). Resting intracellular Ca^{2+} was 126 ± 9 nM while the peak Ca^{2+} was 391 ± 10 nM and the plateau was 26 ± 4 nM above baseline (mean $\pm$ s.e.mean, $n = 26$). Peak levels were unaffected by chelation of extracellular Ca^{2+} with EGTA, but the plateau phase was abolished (Figure 1b). In the absence of extracellular Ca^{2+} , the plateau phase could be reestablished with addition of CaCl_2 (2 mM) after the rapid phase transient (Figure 1c). These data suggest the peak phase represents Ca^{2+} mobilized from intracellular stores while the plateau phase is dependent on the influx of extracellular Ca^{2+} . Examination of Type II cells maintained in primary culture for 18–20 h also revealed a biphasic Ca^{2+} -response to ATP stimulation, but the magnitude and duration of both phases was diminished (not shown).

Figure 2 Dose-response for ATP-stimulated Ca^{2+} mobilization and $[^3\text{H}]$ -phosphatidylcholine ($[^3\text{H}]$ -PC) secretion: Type II cells were either incubated with $[^3\text{H}]$ -choline (18 h) (a) or loaded with Fura 2 after isolation (b) after being suspended by gentle scraping with a rubber policeman. Cells were then exposed to various concentrations of ATP and Ca^{2+} mobilization or $[^3\text{H}]$ -PC release were determined as described in Methods. Results represent the mean of 3 separate experiments done in triplicate and vertical lines show s.e.mean.

Figure 3 Recovery of ATP-stimulated calcium mobilization response: freshly isolated Type II cells were loaded with Fura 2 as noted in Methods then exposed to ATP (1×10^{-5} M) at time 0. At 5, 10, 15, or 30 min a second identical dose of ATP was added. The results shown are representative of 11 separate experiments.

Comparison of dose-response curves for ATP-induced Ca^{2+} mobilization and ATP-stimulated surfactant phospholipid secretion demonstrated similar EC_{50} s (1×10^{-6} M, Figure 2). To determine which phase of Ca^{2+} mobilization was associated with surfactant phospholipid release, EGTA (2 mM) was added to the incubation media 30 min before stimulation with ATP. Basal [^3H]-PC secretion ($1.2 \pm 0.2\%$ of total [^3H]-PC-labelled) was not significantly altered with the addition of EGTA ($2.2 \pm 0.7\%$). ATP significantly elevated [^3H]-PC

secretion to $6.5 \pm 0.3\%$, and the addition of EGTA had no significant effect on ATP-stimulated [^3H]-PC secretion ($6.1 \pm 1.6\%$) (values represent mean $\pm$ s.e. mean of three experiments done in triplicate). These data argue against association of the plateau phase of Ca^{2+} mobilization with [^3H]-PC secretion.

Restimulation of freshly isolated cells following the initial Ca^{2+} -response revealed rapid desensitization of the purinoceptor system. There was

Figure 4 Effect of EGTA on response recovery at ATP-stimulated calcium mobilization: freshly isolated Fura 2 loaded Type II cells were treated with ATP (1×10^{-5} M) in the presence of 2 mM EGTA. At 15 or 30 min, a second identical ATP dose was added. Results shown are representative of 3 separate experiments.

Figure 5 The effect of agonist removal on response recovery: Fura 2 loaded Type II cells were exposed to ATP (1×10^{-5} M) at time 0. During the plateau phase the cell suspension was centrifuged, at $9000g \times 30$ s, supernatant medium containing ATP was removed and cells were resuspended in fresh medium. At 3 min, ATP (1×10^{-5} M) was again added. The results shown are representative of 3 separate experiments.

diminished Ca^{2+} -response to restimulation with ATP at 5 or 10 min after the first exposure (Figure 3). However, by 15 to 30 min following the initial exposure to ATP, the response system was fully recovered (Figure 3). Chelation of extracellular Ca^{2+} with EGTA suppressed recovery at least 30 min (Figure 4). These findings suggest complete recovery from ATP-induced desensitization requires extracellular Ca^{2+} .

To investigate the role of ATP in desensitization, we tested the effect of agonist removal on Ca^{2+} -dependent-response recovery. Following the first ATP-induced Ca^{2+} transient, cells were quickly washed to remove exogenous ATP and resuspended in fresh medium. Re-exposure to ATP 3 min after initial stimulation demonstrated prompt recovery of the Ca^{2+} -response (Figure 5). However, cells initially stimulated and resuspended in medium containing EGTA, demonstrated a blunted Ca^{2+} -response to restimulation with ATP (Figure 6). These findings suggest the presence of agonist desensitizes the Ca^{2+} -response in Type II cells, and removal of agonist results in complete recovery of the response if extracellular Ca^{2+} is present.

Exogenous ATP is removed *in vivo* by cell surface ectoATPases which hydrolyze ATP. Therefore, we tested the hypothesis that a poorly hydrolyzed

Figure 7 Calcium mobilization and response recovery, effect of adenosine 5'-O-(3-thiotriphosphate) (ATP- γ -S): Fura 2 loaded fresh Type II cells in medium containing Ca^{2+} were exposed to ATP- γ -S (1×10^{-5} M) at time 0. At 15 or 30 min following this initial exposure, the cells were exposed to ATP (1×10^{-5} M). The results shown are representative of 3 separate experiments.

Figure 6 Effect of agonist removal and EGTA on response recovery: freshly isolated Fura 2 loaded Type II cells were exposed to ATP (1×10^{-5} M) in the presence of 2 mM EGTA at time 0. When the fluorescence signal had returned to baseline the cell suspension was centrifuged, supernatant removed, and cells were resuspended in fresh Ca^{2+} -containing medium (a), or fresh medium with 2 mM EGTA (b). At 4 min, a second ATP (1×10^{-5} M) dose was added. The results shown are representative of 3 separate experiments.

agonist would prolong the desensitization period. The ATP analogue, ATP- γ -S, stimulated a biphasic Ca^{2+} -response similar to ATP. However, in the continued presence of this poorly hydrolyzed agonist, cells were refractory to ATP stimulation for more than 30 min (Figure 7). These data support the hypothesis that continued presence of agonist desensitizes the Type II cells.

Although ATP-induced Ca^{2+} mobilization was associated with surfactant phospholipid secretion and the Type II cell was capable of multiple peak Ca^{2+} transients, there was no significant difference in surfactant [^3H]-PC release following stimulation with a single $10 \mu\text{M}$ dose of ATP, five $10 \mu\text{M}$ pulses of ATP 30 min apart or a single $50 \mu\text{M}$ ATP dose (Table 1). This suggests an initial ATP-induced Ca^{2+} transient is associated with maximal surfactant phospholipid secretion, since repeated calcium transients are not associated with further surfactant phospholipid secretion.

Discussion

Extracellular ATP stimulated biphasic intracellular Ca^{2+} transients in freshly isolated and cultured alveolar Type II cells. The initial rapid phase was not dependent on extracellular Ca^{2+} , suggesting agonist-stimulated release of Ca^{2+} from intracellular stores. The plateau phase was dependent on extracellular Ca^{2+} levels. These findings are consistent

Table 1 Effect of multiple doses of ATP on [^3H]-phosphatidylcholine ([^3H]-PC) secretion

Agonist dosage	% [^3H]-PC released
Control	1.3 $\pm$ 0.5
10 μM ATP	4.6 $\pm$ 0.6
50 μM ATP	5.5 $\pm$ 0.9
10 μM ATP $\times$ 5 (30 min intervals)	5.9 $\pm$ 1.3

Values are means $\pm$ s.e.mean, $n = 4$.

Release of [^3H]-PC from prelabelled Type II cells was determined after exposure to 10 μM ATP, 50 μM ATP, or 5 doses of 10 μM ATP at 30 min intervals.

[^3H]-PC release was determined as described in Methods, 3 h after initial exposure to agonist.

with ATP-stimulated Ca^{2+} mobilization in smooth muscle cells and Ehrlich ascites tumor cells (Tawada *et al.*, 1987; Dubyak, 1986), where a peak and plateau phase was also noted. The reason for the diminished response of cultured (18 h) cells is unknown, but may represent biochemical changes since Type II cells are known to lose Type II cell morphology following isolation (Cott *et al.*, 1987; Dobbs *et al.*, 1988).

The link between agonist binding and Ca^{2+} release is thought to be myo-inositol 1,4,5 triphosphate (IP_3) created by receptor-mediated hydrolysis of membrane phosphatidyl inositol 4,5-bisphosphate (Williamson, 1986). ATP stimulation of hepatocytes and Ehrlich ascites tumour cells leads to cytosolic elevation of IP_3 (Charest *et al.*, 1985; Dubyak, 1986), and treatment of permeabilized cells with IP_3 mobilizes Ca^{2+} from intracellular stores (Joseph *et al.*, 1984; Dubyak, 1986). Although production of IP_3 continues in the presence of bound agonist (Ambler *et al.*, 1987; Nakahata & Harden, 1987), the transient nature of the Ca^{2+} peak results from the combined effect of Ca^{2+} extrusion by plasma membrane, Ca^{2+} , Mg^{2+} -dependent ATPase, uptake by other cytoplasmic constituents, and Ca^{2+} pool depletion (Putney, 1986; Merritt & Rink, 1987).

The plateau phase seen in Type II cells is dependent on extracellular Ca^{2+} . In Ca^{2+} -free medium, ATP stimulated a rapid Ca^{2+} transient which returned to basal levels within 90 s. In the continued presence of agonist, the plateau phase could be re-established by the addition of 2 mM Ca^{2+} to the medium. This is consistent with the Ca^{2+} -response in carbachol-treated parotid cells (Merritt & Rink, 1987). Moreover, Merritt & Rink (1987) found displacement of carbachol with atropine before the addition of Ca^{2+} prevented the reestablishment of

the plateau phase. This suggests the plateau phase is not only dependent on extracellular Ca^{2+} , but also depends on receptor occupation by agonist. While it is generally accepted that the plateau phase depends on extracellular Ca^{2+} , the mechanism and route of Ca^{2+} influx remains in question. Studies with sea-urchin eggs and from T-lymphocytes suggest a direct influx of Ca^{2+} through transmembrane channels activated by IP_3 (Kuno & Gardner, 1987) and IP_4 (Irvine & Moor, 1986). Putney (1986) and Merritt & Rink (1987) have proposed a more direct pathway between the plasma membrane and endoplasmic reticulum for Ca^{2+} entry to the cytosol. Agonist-stimulated IP_3 production empties the endoplasmic reticulum Ca^{2+} pool. This drop in endoplasmic reticulum Ca^{2+} concentration opens the plasma membrane/endoplasmic reticulum pathway allowing influx of Ca^{2+} directly from the extracellular space to the endoplasmic reticulum, then cytosol, through the IP_3 -activated Ca^{2+} channels (Putney, 1986; Merritt & Rink, 1987). This plateau phase of Ca^{2+} influx continues as long as agonist-stimulated IP_3 production continues. Regardless of pathway, the plateau phase in isolated Type II cells requires extracellular Ca^{2+} and receptor occupancy by agonist.

In ATP-stimulated Type II cells, we found a 15 min refractory period before the Ca^{2+} -response recovered fully. Chelation of extracellular Ca^{2+} suppressed recovery at least 30 min, suggesting that complete recovery from ATP-induced desensitization of the Ca^{2+} -response requires refilling of intracellular Ca^{2+} pools from an extracellular source. This process is similar to that described in smooth muscle (Den Hertog *et al.*, 1985).

The poorly hydrolyzed ATP analogue, ATP- γ -S, produced a refractory period greater than 30 min, suggesting receptor occupancy blocks response recovery. Consistent with this hypothesis, removal of extracellular ATP following the initial transient resulted in prompt response recovery. Similar experiments in the absence of extracellular Ca^{2+} demonstrated refilling of the Ca^{2+} pool occurred within 60 s after Ca^{2+} addition in the absence of agonist. These findings are similar to those in Ehrlich ascites tumour cells (Dubyak, 1986) and hepatocytes (Charest *et al.*, 1985). Charest *et al.* (1985) found an even more rapid recovery of Ca^{2+} -response in hepatocytes compared to Type II cells. Within 3 min, a second ATP dose produced an identical Ca^{2+} transient with less than 20% of the initial ATP remaining after 20 s. These findings suggest the rate limiting step to Ca^{2+} -response recovery is ATP hydrolysis. The difference between hepatocyte response recovery and Type II cell recovery may reflect differences in number of ectoATPases, the rate of ATP hydrolysis by the ATPases, or ATP receptors on each cell type.

The relationship between ATP stimulated Ca^{2+} mobilization and phospholipid secretion in isolated alveolar Type II cells was examined. We found a similar EC_{50} for intracellular Ca^{2+} mobilization and surfactant phospholipid secretion. This is consistent with the findings of Rice & Singleton (1987) where the order of potency among methylene, thio and imido substituted ATP analogues was similar for phospholipid release and Ca^{2+} mobilization in cultured Type II cells. These findings suggest Ca^{2+} mobilization is associated with ATP-stimulated surfactant phospholipid release. While the Type II cell was capable of multiple ATP-induced Ca^{2+} transients following purinoceptor recovery, there was no additional surfactant phospholipid release with sequential ATP stimulation. Extracellular Ca^{2+} depletion abolished the plateau phase of ATP-stimulated Ca^{2+} transients, but had no effect on rapid phase Ca^{2+} peak transients nor surfactant phospholipid secretion. These data suggest a single ATP-stimulated peak Ca^{2+} transient is associated with maximal surfactant phospholipid secretion from Type II cells and may reflect the presence of a pre-formed pool of lamellar bodies which must be regenerated prior to the next secretory stimulus. Alternatively, other steps in the signal pathway may be down-regulated following the initial secretory stimulus.

Although investigations with phorbol esters have shown elevation of cytoplasmic free Ca^{2+} is not a necessary event in surfactant phospholipid secretion

(Sano *et al.*, 1985), a synergistic effect on secretion has been demonstrated between phorbol esters and A23187 which elevates intracellular Ca^{2+} in Type II cells (Dobbs *et al.*, 1986b). This suggests complementary pathways for surfactant phospholipid secretion in Type II cells. Evidence in other cell types suggests ATP may mobilize intracellular Ca^{2+} and activate protein kinase C. In hepatocytes, ATP stimulates hydrolysis of phosphatidyl inositol 4,5 biphosphate (PIP_2) to generate IP_3 (Charest *et al.*, 1985). The other product of agonist-stimulated PIP_2 hydrolysis is diacylglycerol (DAG), a potent activator of protein kinase C (Berridge, 1987). Although it has yet to be demonstrated in Type II cells, the ATP-induced generation of both IP_3 and DAG may create the secretory synergism of Ca^{2+} mobilization and protein kinase C activation seen in previous studies of surfactant phospholipid release.

In summary, while the final common pathway for surfactant phospholipid secretion from Type II cells is not known, initial binding of extracellular ATP appears sufficient to mobilize intracellular Ca^{2+} and stimulate phospholipid secretion. Recovery of the P_2 -purinoceptor-stimulated Ca^{2+} transient is dependent on agonist removal and presence of extracellular Ca^{2+} .

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References

- AMBLER, S.K., THOMPSON, B., SOLSKI, P.A., BROWN, J.H. & TAYLOR, P. (1987). Receptor-mediated inositol phosphate formation in relation to calcium mobilization: a comparison of two cell lines. *Mol. Pharmacol.*, **32**, 376–383.
- BERRIDGE, M.J. (1987). Inositol triphosphate and diacylglycerol: two interacting second messengers. *Ann. Rev. Biochem.*, **56**, 159–193.
- CHAPAL, J. & LOUBATIÈRES-MARIANI, M. (1981). Effects of phosphate-modified adenine nucleotide analogs on insulin secretion from perfused rat pancreas. *Br. J. Pharmacol.*, **73**, 105–110.
- CHAREST, R., BLACKMORE, P.F. & EXTON, J.H. (1985). Characterization of responses of isolated rat hepatocytes to ATP and ADP. *J. Biol. Chem.*, **260**, 15789–15794.
- COTT, G.R., WALKER, S.R. & MASON, R.J. (1987). The effect of substratum and serum on the lipid synthesis and morphology of alveolar Type II cells *in vitro*. *Exp. Lung Res.*, **13**, 427–447.
- DEN HERTOOG, A., PIELKENROOD, J. & VEN DEN AKKER, J. (1985). Effector mechanisms for α,β -methylene ATP and ATP derivatives in guinea pig taeni caeci. *Eur. J. Pharmacol.*, **110**, 95–101.
- DOBBS, L.G., GONZALEZ, R. & WILLIAMS, M.C. (1986a). An improved method for isolating Type II cells in high yield and purity. *Am. Rev. Respir. Dis.*, **134**, 141–145.
- DOBBS, L.G., GONZALEZ, R.F., MARINARI, L.A., MESCHER, E.J. & HAWGOOD, S. (1986b). The role of calcium in the secretion of surfactant by rat alveolar Type II cells. *Biochim. Biophys. Acta*, **877**, 305–313.
- DOBBS, L.G., WILLIAMS, M.C. & GONZALEZ, R. (1988). Monoclonal antibodies specific to apical surfaces of rat alveolar Type I cells bind to surfaces of cultured, but not freshly isolated, Type II cells. *Biochim. Biophys. Acta*, **970**, 146–156.
- DORN, C.C. & RICE, W.R. (1988). Regulation of surfactant phospholipid secretion: calcium mobilization and response recovery following P_2 -purinoceptor stimulation in freshly isolated alveolar Type II cells. *Pediatr. Res.*, **23**, 241A.
- DUBYAK, G.R. (1986). Extracellular ATP activates polyphosphoinositide breakdown and Ca^{2+} mobilization in Ehrlich ascites tumor cells. *Arch. Biochem. Biophys.*, **245**, 84–95.
- FOLCH, J., LEES, M. & SLOAN-STANLEY, G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.*, **226**, 497–509.

- GALLACHER, D.V. (1982). Are there purinergic receptors on parotid acinar cells? *Nature*, **296**, 83–86.
- GILFILLAN, A.M. & ROONEY, S.A. (1988). Functional evidence for involvement of P₂-purinoceptors in the ATP stimulation of phosphatidylcholine secretion in Type II alveolar epithelial cells. *Biochim. Biophys. Acta*, **959**, 31–37.
- GORDON, J.L. (1986). Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**, 309–319.
- HAUSSINGER, D., STEHLE, T., GEROK, W., TRAN-THI, T. & DECKER, K. (1987). Hepatocyte heterogeneity in response to extracellular ATP. *Eur. J. Biochem.*, **169**, 645–650.
- IRVINE, R.F. & MOOR, R.M. (1986). Micro-injection of inositol 1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external Ca²⁺. *Biochem. J.*, **240**, 917–920.
- JOSEPH, S.K., THOMAS, A.P., WILLIAMS, R.J., IRVINE, R.F. & WILLIAMSON, J.R. (1984). myo-Inositol 1,4,5-triphosphate: a second messenger for the hormonal mobilization of intracellular Ca²⁺ in liver. *J. Biol. Chem.*, **259**, 3077–3081.
- KUNO, M. & GARDNER, P. (1987). Ion channels activated by inositol 1,4,5-triphosphate in plasma membrane of human T-lymphocytes. *Nature*, **326**, 301–304.
- LEFKOWITZ, R.J. & CARON, M.G. (1986). Regulation of adrenergic receptor function by phosphorylation. *Curr. Top. Cell. Reg.*, **28**, 209–231.
- MCDONOUGH, P.M., EUBANKS, J.H. & BROWN, J.H. (1988). Desensitization and recovery of muscarinic and histaminergic Ca²⁺ mobilization in 1321N1 astrocytoma cells. *Biochem. J.*, **249**, 135–141.
- MERRITT, J.E. & RINK, T.J. (1987). Regulation of cytosolic free calcium in fura 2-loaded rat parotid acinar cells. *J. Biol. Chem.*, **262**, 17362–17369.
- NAKAHATA, N. & HARDEN, T.K. (1987). Regulation of inositol triphosphate accumulation by muscarinic cholinergic and H₁-histamine receptors on human astrocytoma cells. *Biochem. J.*, **241**, 337–344.
- PRPIĆ, V., BLACKMORE, P.F. & EXTON, J.H. (1982). Myo-inositol uptake and metabolism in isolated rat liver cells. *J. Biol. Chem.*, **257**, 11323–11331.
- PUTNEY, J.W., JR (1986). A model for receptor-regulated calcium entry. *Cell Calcium*, **7**, 1–12.
- RICE, W.R. & SINGLETON, F.M. (1986). P₂-purinoceptors regulate surfactant secretion from rat isolated alveolar Type II cells. *Br. J. Pharmacol.*, **89**, 485–491.
- RICE, W.R. & SINGLETON, F.M. (1987). P₂-purinoceptor regulation of surfactant secretion from rat isolated alveolar Type II cells is associated with mobilization of intracellular calcium. *Br. J. Pharmacol.*, **91**, 833–838.
- RICE, W.R., OSTERHOUDT, K.C. & WHITSETT, J.A. (1984). Effect of cytochalasins on surfactant release from alveolar Type II cells. *Biochim. Biophys. Acta*, **805**, 12–18.
- SANO, K., VOELKER, D.R. & MASON, R.J. (1985). Involvement of protein kinase C in pulmonary surfactant secretion from alveolar Type II cells. *J. Biol. Chem.*, **260**, 12725–12729.
- SIBLEY, D.R. & LEFKOWITZ, R.J. (1985). Molecular mechanisms of receptor desensitization using the β -adrenergic receptor-coupled adenylate cyclase system as a model. *Nature*, **317**, 124–129.
- TAWADA, Y., FURUKAWA, K. & SHIGEKAWA, M. (1987). ATP-induced calcium transient in cultured rat aortic smooth muscle cells. *J. Biochem.*, **102**, 1499–1509.
- TOEWS, M.L., WALDO, G.L., HARDEN, T.K. & PERKINS, J.P. (1984). Relationship between altered membrane form and a low affinity form of the β -adrenergic receptor occurring during catecholamine-induced desensitization. *J. Biol. Chem.*, **259**, 11844–11850.
- WILLIAMSON, J.R. (1986). Role of inositol lipid breakdown in the generation of intracellular signals. *Hypertension*, **8**, suppl. II, 140–156.
- ZAWALICH, W., BROWN, C. & RASMUSSEN, H. (1983). Insulin secretion: combined effects of phorbol ester and A23187. *Biochem. Biophys. Res. Commun.*, **117**, 448–455.

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