Cellular activation by Ca^{2+} release from stores in the endoplasmic reticulum but not by increased free Ca^{2+} in the cytosol

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Ca^{2+} release from intracellular stores and/or transmembrane influx can increase the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Such changes in $[\text{Ca}^{2+}]_i$ might transduce signals regulating transcription, motility, secretion, and so on. Surfactant secretagogues such as ATP and ionomycin stimulate the release and transmembrane influx of Ca^{2+} , both of which increase $[\text{Ca}^{2+}]_i$. The addition of surfactant protein A (SP-A) or depleting cellular Ca^{2+} inhibited both surfactant secretion and Ca^{2+} transients. Current results suggest that Ca^{2+} signalling stimulates surfactant secretion by type II pneumocytes, but not via increased $[\text{Ca}^{2+}]_i$. Treatment of cells with a Ca^{2+} chelator, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid acetoxymethyl ester (BAPTA-AM), stimulated secretion but decreased $[\text{Ca}^{2+}]_i$. Adding SP-A or depleting Ca^{2+} inhibited BAPTA-AM-induced secretion. When studied directly, Ca^{2+} in the endoplasmic reticulum

store ($[\text{Ca}^{2+}]_r$) decreased in response to BAPTA, ionomycin and thapsigargin, and increased in response to SP-A. Phorbol ester (PMA) induced surfactant secretion without altering $[\text{Ca}^{2+}]_i$ or $[\text{Ca}^{2+}]_r$ and was unaffected by Ca^{2+} depletion. The addition of PMA to Ca^{2+} -releasing secretagogues increased secretion, but combining two Ca^{2+} -releasing secretagogues did not. These results suggest that (1) Ca^{2+} signalling of type II cell surfactant secretion reflects changes in $[\text{Ca}^{2+}]_r$, not $[\text{Ca}^{2+}]_i$, (2) PMA elicits secretion differently from Ca^{2+} -releasing secretagogues, and (3) SP-A inhibits secretion by enhancing Ca^{2+} sequestration within endoplasmic reticulum stores. Whether other cell types signal via changes in $[\text{Ca}^{2+}]_i$ is unknown.

Key words: Ca^{2+} channels, endoplasmic reticulum Ca^{2+} stores, surfactant, type II pneumocytes.

INTRODUCTION

Surfactant is a mixture of lipids and associated proteins that is manufactured, stored and secreted by type II alveolar pneumocytes. It is essential for normal respiratory function. Surfactant insufficiency, whether due to prematurity, inactivation or genetic deficiency, is not compatible with extra-uterine life [1–3]. The mechanism(s) that activate and regulate surfactant secretion are not well understood but evidence suggests that Ca^{2+} is important in signalling this process. Here we report that the role of Ca^{2+} signalling to activate surfactant secretion reflects changes in the concentration of free Ca^{2+} in cytosol stores (luminal Ca^{2+} concentration, or $[\text{Ca}^{2+}]_r$) and is independent of the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$).

Surfactant secretion by type II cells is often studied *in vitro*, using receptor-binding secretagogues such as purines or β -adrenergic agonists [4–7]. Surfactant secretion can also be activated independently of cell membrane receptors, for example by Ca^{2+} ionophores such as ionomycin (Io). With or without receptor mediation, these secretagogues elicit rapid, large changes in $[\text{Ca}^{2+}]_i$; Ca^{2+} release from intracellular stores is followed by influx through plasma membrane Ca^{2+} channels [7,8]. The importance of Ca^{2+} signalling in surfactant secretion is underscored by the fact that thapsigargin (TG), which inhibits Ca^{2+} -dependent ATPase in the endoplasmic reticulum (ER), and therefore acts directly on stores to increase $[\text{Ca}^{2+}]_r$, also stimulates surfactant secretion [7,9].

Phorbol esters, which are potent surfactant secretagogues, are the exception. They stimulate surfactant secretion without altering $[\text{Ca}^{2+}]_i$ [4].

One of the surfactant proteins, surfactant-associated protein A (SP-A), decreases secretagogue-stimulated surfactant secretion [6,10–13]. SP-A binds a type II cell membrane receptor (SP-A receptor; SPAR) to prevent the Ca^{2+} release elicited by all the above secretagogues, including those acting directly on stores (Io and TG) [7]. SP-A does not block transmembrane Ca^{2+} fluxes, whether active (i.e. via channels gated by voltage or by other means) or passive (e.g. via Io [7]).

The signalling mechanisms that stimulate and inhibit surfactant secretion are not well understood. The diversity of secretagogues, some with different receptors and some without receptors but each with separate signalling pathways, greatly complicates the task of elucidating how surfactant secretion is triggered [14,15]. The nature of the signal that begins at SPAR and down-regulates surfactant secretion is even more obscure.

Adenosine binds A_2 purine receptors to activate adenylate cyclase via G-protein-dependent pathways to produce cAMP. cAMP activates protein kinase A. β -Adrenergic agonists act similarly, although through different receptors. ATP binds P_{2y} and P_{2u} receptors to signal via G-proteins to activate phospholipase C_β (phosphoinositide-specific phospholipase C), which hydrolyses PtdIns(1,4,5) P_3 to diacylglycerol and Ins(3,4,5) P_3 , both of which activate other enzymes such as protein kinases C and phospholipase D. Secretagogue-induced signalling in type II cells downstream of these points is poorly understood.

Intracellular Ca^{2+} stores in different cell types possess several types of receptor that can be stimulated to cause Ca^{2+} release [8,16]. Ryanodine and Ins(1,4,5) P_3 receptors [Ins(1,4,5) P_3 R] are examples of proteins that traverse the ER membrane and release Ca^{2+} on binding their ligands. Ca^{2+} release (or increased $[\text{Ca}^{2+}]_r$)

Abbreviations used: BAPTA-AM, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid acetoxymethyl ester; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; $[\text{Ca}^{2+}]_r$, free $[\text{Ca}^{2+}]$ in ER stores (luminal); ER, endoplasmic reticulum; Io, ionomycin; Ins(1,4,5) P_3 R, Ins(1,4,5) P_3 receptor; MEM, minimal essential medium; PL, phospholipid; SP-A, surfactant-associated protein A; SPAR, SP-A receptor; TG, thapsigargin; TPEN, *N,N,N',N'*-tetrakis-(2-pyridylmethyl) ethylenediamine.

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can activate tyrosine-specific protein kinases and calmodulin-dependent kinases [17]. Again, however, the means by which this signalling mechanism would result in surfactant secretion is unclear.

Downstream signalling that follows Ca^{2+} release from stores is an area of intense investigation. In type II cells, Ca^{2+} release activates cell membrane Ca^{2+} channels, allowing the influx of Ca^{2+} [18–20]. (It is not known whether type II cells bear P_{2x} receptors, which are themselves Ca^{2+} channels.) The nature of the stimulus or stimuli that open Ca^{2+} channels is under debate: candidates range from increased $[\text{Ca}^{2+}]_i$ to poorly characterized substances that might be released simultaneously with Ca^{2+} from those stores [8].

Ca^{2+} ionophores are surfactant secretagogues that lack plasma membrane receptors. They release Ca^{2+} from ER stores directly and carry Ca^{2+} from outside the cell into the cytosol. Our recent report of secretagogue activity for TG, which directly inhibits the pump that maintains the Ca^{2+} gradient at the ER stores, underscores the importance of ER Ca^{2+} stores in signalling surfactant secretion [6].

This report examines the role of Ca^{2+} in signalling surfactant secretion. We undertook these studies to clarify the parallel abilities of SP-A to inhibit surfactant secretagogue activities and Ca^{2+} transients, not only for secretagogues that act through cell membrane receptors but also for secretagogues that release Ca^{2+} directly (Io and TG). The findings reported here suggest a novel role for Ca^{2+} stores in cellular activation.

MATERIALS AND METHODS

Animals

Specified-pathogen free, female Sprague–Dawley rats (180–200 g) were purchased from Charles River Laboratories. The animals were used as sources of alveolar type II cells within 1 week of receipt.

Chemicals and reagents

SP-A from bovine lung was purified and analysed as described [21]. Bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid acetoxymethyl ester (BAPTA-AM) and *N,N,N',N'*-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) were purchased from Molecular Probes (Corvallis, OR, U.S.A.). (To avoid confusion, we shall use the term 'BAPTA-AM' in this paper to denote the esterified form of BAPTA, which is placed in the culture medium and with which the cells are loaded. The intracellular chelator will be referred to as 'BAPTA'.) After initial dose–response studies, 25 μM BAPTA-AM was used to load type II cells.

Type II cell cultures

Freshly isolated type II cells were prepared by standard techniques, originally described by Dobbs et al. [21] and later modified by Sen and Chander [5]. We have reported the use of these techniques previously [6,7]. In brief, lungs of anaesthetized rats were cleared of blood and treated with elastase endotracheally (Worthington Biochemical, Freehold, NJ, U.S.A.) to obtain free cells. The cells were separated from lung debris and plated on bacteriological plates coated with normal rat IgG (Sigma Chemical Co., St. Louis, MO, U.S.A.). After 1 h, free cells were collected by panning; they were then pelleted and resuspended (8×10^5 cells/ml) in minimum essential medium (MEM)/10% (v/v) fetal-calf serum (Life Technologies, Gaithersburg, MD, U.S.A.).

Secretion studies

Secretion studies were performed as described by Sen and Chander [5]. In brief, 8×10^5 alveolar type II cells were cultured for 18 h in MEM/10% (v/v) fetal-calf serum containing 0.5 μCi of [*methyl*- ^3H]choline (Amersham, Arlington Heights, IL, U.S.A.) to label cellular phospholipids (PLs) metabolically. Cells were washed to remove non-adherent cells and unincorporated radioactivity. After replacing this medium with fresh MEM, plates were incubated for 15 min. SP-A (100 ng/ml) was added to selected plates, followed by incubation for a further 15 min; secretagogues were then added at the appropriate concentrations [control (no addition), ATP (500 μM), TG (10 μM), Io (1 μM), PMA (50 ng/ml)]. At this point (zero time) in each experiment, some samples were removed for the analysis of secreted labelled lipids to establish a baseline. Incubation proceeded for a further 2 h for all other plates. After this incubation, media were removed and centrifuged (15 min, 300 *g*) to pellet cells that detached during incubation. These cells were later pooled with those recovered from the plates. Lipids from both media and plates were extracted [5] after adding [*methyl*- ^{14}C]dipalmitoyl phosphatidylcholine (Amersham) as recovery standard, and egg phosphatidylcholine (Sigma) as a carrier lipid. Recovered lipids were dissolved in Scintiverse II fluid (Fisher Scientific, Pittsburgh, PA, U.S.A.) and the radioactivity of these lipid extracts was measured with a scintillation counter (Beckman Instruments, Fullerton, CA, U.S.A.). The recovery of ^3H -labelled PLs was normalized to the recovery of [*methyl*- ^{14}C]dipalmitoyl phosphatidylcholine. PL secretion was determined as percentage secretion: $100 \times (\text{radioactivity in lipids recovered from media}) / (\text{radioactivity in lipids recovered from media and plates})$.

In some of our studies, involving the effect of Ca^{2+} depletion on PL synthesis, the synthesis of glycerol-based lipids was also measured by quantifying the incorporation of [^3H]glycerol into surfactant lipids. Cell culture and lipid extraction were performed similarly to those described above except that [*1,2,3*- ^3H]glycerol (NEN Life Science Products, Boston, MA, U.S.A.) was used instead of [*methyl*- ^3H]choline.

Type II cell viability assays

Viability of type II cells was tested both by Trypan Blue exclusion and by tetrazolium/formazan assay (Promega Corp., Madison, WI, U.S.A.), which measures mitochondrial dehydrogenases. These enzymes are present in active form only in living cells and are absent from non-viable cells [22]. For such studies, an assay solution containing the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphonyl)-(2*H*)-tetrazolium, inner salt; MTS] and an electron-coupling reagent (phenazine methosulphate) in MEM is added to the cells. MTS is bioreduced by dehydrogenase enzymes found in metabolically active cells into a formazan compound that is soluble in tissue-culture medium and is directly measurable from its absorbance at 490 nm. Correction was made for the small effect of BAPTA itself on this reaction by running a parallel assay on plates without cells.

Measurements of intracellular [Ca^{2+}]

Freshly isolated type II cells (10^6) were added to 35 mm tissue culture plates, each containing a polylysine (Sigma)-coated 25 mm diameter coverslip and incubated [37°C , air/ CO_2 (19:1)] for at least 2 h; 20 min before use, fura-2 acetoxymethyl ester (Molecular Probes) was added (final concentration 3 μM) to each coverslip. After being loaded with fura-2, the coverslips were washed with cell incubation buffer [121 mM NaCl/4.7 mM

KCl/1.2 mM KH₂PO₄/1.2 mM MgSO₄/2 mM CaCl₂/10 mM glucose/5 mM NaHCO₃/0.2 % BSA/10 mM Hepes/NaOH (pH 7.4)] and inserted into a thermostatically regulated chamber (37 °C). Cell incubation buffer was added and the whole assembly was placed on the stage of a Nikon Diaphot inverted microscope. At appropriate times (see the Results section), secretagogues in the appropriate concentration (see above) were added. Fluorescence images of the cells were collected by exciting the cells alternately at 340 and 380 nm and measuring the emission at 500 nm with a charge-coupled device camera (Photometrics) as described previously [23]. Image acquisition and analysis were done with Macintosh computers running customized image-processing software. The ratio of fura-2 excitation at 340 and 380 nm was calibrated to [Ca²⁺]_i as described [23,24].

Ca²⁺ concentration in the ER [25]

Type II cells were prepared as described and plated on glass coverslips that had been pretreated with poly-(L-lysine) (Sigma) (0.01 %, 20 °C, 15 min). After incubation overnight at 37 °C in MEM/10 % (v/v) fetal-calf serum, the cells were washed three times with serum-free MEM and once with loading buffer [121 mM NaCl/4.7 mM KCl/1.2 mM KH₂PO₄/1.2 mM MgSO₄/2 % (w/v) BSA/10 mM glucose/20 mM Hepes/2 mM CaCl₂]. The cells were loaded in loading buffer containing 10 μ M fura-2ff acetoxymethyl ester (Teflabs, Austin, TX, U.S.A.) and 0.06 % Pluronic Acid (Molecular Probes) for 2 h at 37 °C, with shaking. The coverslip was washed in Ca²⁺-free imaging buffer (121 mM NaCl/4.7 mM KCl/1.2 mM KH₂PO₄/1.2 mM MgSO₄/10 mM glucose/20 mM Hepes), inserted into a thermostatically regulated chamber (37 °C), and mounted on an inverted fluorescence microscope in 1 ml of imaging buffer. Fluorescence imaging after excitation at 340 and 380 nm was performed as described above.

During imaging MnCl₂ was added at zero time to a final concentration of 500 μ M followed by SP-A, Io or TG (final concentrations 100 ng/ml, 1 μ M and 4 μ M respectively).

RESULTS

Phorbol ester treatment does not alter cytosol Ca²⁺

We examined the effect of the phorbol ester secretagogue PMA on [Ca²⁺]_i. We have reported that ATP, terbutaline, Io and TG all elicited biphasic increases in [Ca²⁺]_i, reflecting both Ca²⁺ release and Ca²⁺ influx [7]. Like the secretagogue activity of these compounds, increases in [Ca²⁺]_i were inhibited by SP-A, acting through its type II cell membrane receptor. The lack of alteration in [Ca²⁺]_i on the treatment of type II cells with PMA has been reported [4,26]. We tested the effect of PMA on type II cell [Ca²⁺]_i by digital imaging fluorescence microscopy and confirmed that PMA did not alter [Ca²⁺]_i in type II cells (results not shown).

Ca²⁺ depletion decreases the surfactant secretion induced by Io and TG but not that induced by PMA

Both the surfactant secretion and Ca²⁺ transients stimulated by ATP, Io and TG are inhibited by SP-A [7]. PMA, in contrast, stimulates surfactant secretion without altering [Ca²⁺]_i. To test whether secretion induced by these secretagogues required Ca²⁺ signalling, we studied secretagogue activities in Ca²⁺-depleted type II cells.

Type II cells were incubated overnight and loaded with [³H]PL precursor in Ca²⁺-free medium. They were then treated with ATP, Io, TG or PMA, still in Ca²⁺-free medium, and PL secretion was measured 2 h later. Ca²⁺ depletion in this manner prevented PL secretion stimulated by ATP and Io, and partly

Figure 1 Secretagogue-induced surfactant secretion with and without Ca²⁺

Type II cell secretion of ³H-labelled PLs was measured as described in the Materials and methods section, as described by Dobbs [21] and Sen and Chander [5]. Cells were either cultured without added secretagogue, or with ATP, Io, PMA or TG. Culture medium was maintained (1) with normal Ca²⁺ concentration (1.64 mM) throughout the experiment (wavy hatched columns), (2) without Ca²⁺ throughout the experiment (white columns) and (3) without Ca²⁺ overnight to deplete cytosol Ca²⁺ stores, but with Ca²⁺ added back to its normal concentration in MEM (1.64 mM) at the start of the 2 h secretion assay (grey columns). Surfactant secretions are shown as percentages of total labelled PLs (means ± S.E.M. for nine independent experiments). **P* < 0.05, compared with both group 1 and group 3 (Actual values are as follows: for group 1 compared with group 2: Io, *P* = 0.0016; for TG, *P* = 0.029; for group 2 compared with group 3: Io, *P* = 0.037; TG, *P* = 0.016). For ATP, the difference between groups 1 and 2 was significant (*P* < 10⁻⁴). Abbreviation: N.D., not done. For PMA, the difference between group 1 and 2 was not statistically significant (*P* = 0.26); neither was that between groups 2 and 3 (*P* = 0.11).

Table 1 Effect of Ca²⁺ depletion on PL synthesis

Type II cells were isolated as indicated in the Materials and methods section for standard surfactant secretion assays, in which either [³H]choline or [1,2,3-³H]glycerol was used as the PL precursor. Cells were cultured overnight with or without Ca²⁺ in the tissue culture medium; surfactant lipids were extracted on the next day. The incorporations of radiolabelled choline and glycerol into surfactant lipids are expressed as percentages of the control (+ Ca²⁺) (means ± S.D.). *P* values were determined by Wilcoxon Signed Rank Test.

PL precursor used	Incorporation into PL (% of control)		
	+ Ca ²⁺	— Ca ²⁺	<i>P</i>
[³ H]choline	100% ± 58%	160.8% ± 38.5%	0.210
[1,2,3- ³ H]glycerol	100% ± 12%	93.0% ± 14.5%	0.249

inhibited that stimulated by TG. It did not alter PMA-stimulated surfactant secretion (Figure 1).

We tested the possibility that the altered PL labelling caused by incubating cells without Ca²⁺ could account for the observed changes. Surfactant secretion is expressed, relative to internal standards, as a percentage of total PLs secreted in 2 h. Thus altered labelling efficiency should not cause observed decreases in secretion in Ca²⁺-depleted cells. To assess the effects on PL synthesis of depleting cells of Ca²⁺, we compared labelled PLs (radioactivity in [³H]choline or [³H]glycerol incorporated into PLs per 8 × 10⁵ cells) in type II cells cultured with and without Ca²⁺ overnight. Culturing overnight without Ca²⁺ did not affect PL synthesis (Table 1).

Figure 2 Effects of BAPTA and TPEN on surfactant secretion by type II cells

Type II cells were isolated as indicated in the Materials and methods section for standard surfactant secretion assays. To these cultures were added different concentrations of BAPTA (■) and TPEN (◆); surfactant secretions are shown as percentages of ^3H -labelled PL secreted (means $\pm$ S.D.) as described in the Materials and methods section. The viability of type II cells (○) was measured by the method of Cory et al. [22] in BAPTA-AM-treated cultures and is shown as a function of BAPTA concentration. * $P < 0.001$, compared with untreated controls and with TPEN-treated cultures.

Secretagogue activity is restored by adding Ca^{2+}

The importance of Ca^{2+} in the activity of these secretagogues was studied in Ca^{2+} -depleted cells by replenishing Ca^{2+} , incubating the cells for 15 min and then adding secretagogues (Figure 1). Adding Ca^{2+} back in this way restored PL secretion in response to Io and TG. Re-adding Ca^{2+} also increased PMA-induced secretion but this effect was not statistically significant.

Before the addition of secretagogues, [^3H]choline-containing medium was removed and the cells were washed. During the 2 h between the re-adding of Ca^{2+} and assay, enhanced PL synthesis from the small pool of unincorporated [^3H]choline within cells might account for the apparently increased secretion. We therefore compared total ^3H -labelled PLs after incubation overnight to that after a further 2 h of culture, both without secretagogue. The additional 2 h during the assay did not increase PL synthesis significantly [207175 ± 78555 (S.D.) c.p.m. overnight, compared with 203554 ± 73101 c.p.m. overnight plus 2 h; $P = 0.640$ by Wilcoxon signed rank test].

Chelation of intracellular Ca^{2+} stimulates surfactant secretion

We have shown above that Ca^{2+} depletion inhibited PL secretion that was induced by Ca^{2+} -releasing secretagogues, and that secretagogue activity was restored by re-adding Ca^{2+} . Secretagogue-induced changes in $[\text{Ca}^{2+}]_i$ might thus be central to surfactant secretion, i.e. increased $[\text{Ca}^{2+}]_i$ due to release from ER stores could signal type II cell activation to secrete PLs. We therefore tested the effects of chelating Ca^{2+} on surfactant secretion. BAPTA-AM, an esterified EGTA analogue, does not bind Ca^{2+} and crosses cell membranes freely. It is de-esterified in the cytosol, whereupon it binds Ca^{2+} . If increases in $[\text{Ca}^{2+}]_i$ elicited by Ca^{2+} -releasing secretagogues were important in PL secretion, BAPTA should decrease surfactant secretion. However, we found that BAPTA is a powerful secretagogue (Figure 2).

BAPTA chelates Ca^{2+} and other cations. TPEN is a chelator of heavy-metal cations. It crosses cell membranes but binds Ca^{2+}

Table 2 Effect of SP-A on BAPTA-induced surfactant secretion

Type II cells were isolated as indicated in the Materials and methods section for standard surfactant secretion assays. To these cultures was added BAPTA (25 μM), with or without SP-A (100 ng/ml); surfactant secretions were measured as percentages of ^3H -labelled PL secreted (means $\pm$ S.E.M.) as described in the Materials and methods section. * $P < 0.02$, compared with cultures treated with BAPTA alone.

Addition	PL secreted (%)
None	8.33 ± 0.15
SP-A	4.98 ± 0.94
BAPTA-AM	15.47 ± 1.24
BAPTA-AM + SP-A	$12.98 \pm 0.61^*$

much more weakly than BAPTA. To test whether the chelation of other cations explains BAPTA secretagogue activity, we tested TPEN in parallel. TPEN showed no secretagogue activity, even at 150 μM (Figure 2).

In these studies, cellular viability was determined in two ways. Viable cells were enumerated by counting Trypan Blue-excluding cells. Neither BAPTA-AM nor TPEN at these concentrations had any effect on cell viability, compared with each other or with cultures of untreated type II cells. In addition, we measured cellular viability in BAPTA-AM-treated cultures with the tetrazolium/formazan assay for mitochondrial dehydrogenase [22]. These results, included in Figure 2 (○), also indicate that the viability of BAPTA-AM-treated type II cells does not differ from that of control cells.

BAPTA-induced PL secretion is inhibited by SP-A

SP-A inhibits the secretagogue activities of ATP, Io and TG, all of which release Ca^{2+} from ER stores [6,7], as well as that of PMA, which does not alter $[\text{Ca}^{2+}]_i$ [27,28]. BAPTA is a powerful secretagogue that binds cytosol Ca^{2+} , buffering $[\text{Ca}^{2+}]_i$. We tested whether SP-A altered BAPTA-induced surfactant secretion. Type II cells were pretreated with SP-A, then exposed to BAPTA-AM (loading concentration 25 μM). After treatment with SP-A, BAPTA induced significantly less surfactant secretion than in control cells that were not exposed to SP-A (Table 2).

BAPTA elicits surfactant secretion by chelating Ca^{2+}

To ascertain that the chelation of Ca^{2+} , and not that of other cations, was responsible for BAPTA secretagogue activity, type II cells were maintained in Ca^{2+} -free medium overnight, and then during and after treatment with BAPTA-AM (25 μM). Depletion of cell Ca^{2+} prevented BAPTA secretagogue activity (Figure 3). Secretagogue activity was restored by re-adding Ca^{2+} after overnight depletion. Therefore, like secretagogues that release Ca^{2+} from stores, BAPTA-induced surfactant secretion requires Ca^{2+} .

Ca^{2+} influx is not necessary for secretagogue activity

Ca^{2+} signalling in many cell types involves both release from stores and transmembrane influx. To test whether Ca^{2+} influx is important in Ca^{2+} signalling of surfactant secretion, we cultured type II cells in standard MEM containing 1.5 mM Ca^{2+} overnight during PL labelling. This medium was exchanged for Ca^{2+} -free medium just before the addition of secretagogues. Despite some variability, most probably due to the assaying of secretion in non-equilibrium conditions, when Ca^{2+} influx was prevented, i.e. by removing extracellular Ca^{2+} during the assay, the surfactant

Figure 3 BAPTA-induced surfactant secretion is inhibited by depleting cells of Ca²⁺ and is restored by adding Ca²⁺ back

Type II cells were isolated as indicated in the Materials and methods section for standard surfactant secretion assays. Cultures were maintained under three conditions: (1) at normal Ca²⁺ concentration (1.64 mM) throughout the study, (2) totally without Ca²⁺ and (3) without Ca²⁺ overnight to deplete cytosol Ca²⁺ stores, but with Ca²⁺ added back to 1.64 mM at the start of the 2 h secretion assay. To these cultures, BAPTA-AM (25 μ M) was added, with or without SP-A (100 ng/ml), and surfactant secretions are shown as percentages of ³H-labelled PL secreted (means $\pm$ S.E.M.), as described in the Materials and methods section. Surfactant secretion was significantly different for cultures receiving BAPTA (grey columns) [compared with controls (wavy hatched columns)] when cells were exposed to Ca²⁺ throughout, or when Ca²⁺ was added back after overnight Ca²⁺ depletion: $P = 0.0001$ and $P = 0.0004$ respectively. Surfactant secretion was not significantly different in BAPTA-AM-treated compared with control cultures incubated totally without Ca²⁺ ($P = 0.496$).

Figure 4 Effects of short-term removal of Ca²⁺ from the culture medium on surfactant secretion by type II cells

Type II cells were isolated as indicated in the Materials and methods section for surfactant secretion assays. To these cultures, different secretagogues were added and cells were cultured overnight normally. For the 2 h secretion assay, cells were exposed to one of the several secretagogues listed, either with or without Ca²⁺ in the medium for the 2 h assay period. Surfactant secretions are shown as percentages of ³H-labelled PL secreted (means $\pm$ S.E.M.), as described in the Materials and methods section. Differences in surfactant secretion between cells cultured in (1) normal medium throughout (wavy hatched columns) and (2) cells cultured overnight with normal medium, followed by 2 h in Ca²⁺-free medium during the assay (white columns) were not significant for control groups or for the Io or BAPTA experimental groups. * $P = 0.03$ comparing groups 1 and 2, by Wilcoxon signed rank test.

secretion elicited by BAPTA or Io was not significantly altered (Figure 4). Statistically significant but small decreases in TG- and PMA-induced secretion were noted.

Figure 5 Surfactant secretion in response to combinations of secretagogues

Type II cells were isolated, surfactant PLs were labelled, and surfactant secretions were assayed as indicated in the Materials and methods section. To these cultures we added BAPTA, Io, TG and PMA, alone or in combination. Type II cells cultured with some combinations of secretagogues (BAPTA + PMA, BAPTA + TG) did not survive the assay period; results shown are for those cultures in which viability exceeded 95% of the originally plated cells. * $P < 0.05$, compared with either secretagogue individually.

Figure 6 Effect of BAPTA on [Ca²⁺]_i

Changes in [Ca²⁺]_i were calculated from the ratio of fura-2 fluorescence induced at 340 nm (fura-2 bound to Ca²⁺), to that induced at 380 nm (unbound), as described in the Materials and methods section. In these representative tracings, cells were preloaded with fura-2 and BAPTA was added at the time indicated (upper panel) or cells were untreated (lower panel).

Secretagogue activities of Ca²⁺-releasing secretagogues are not additive, except to that of PMA

If the several secretagogues stimulate surfactant secretion via the same pathway, their activities should overlap and their secretagogue activities should not be additive. If, however, two

Figure 7 Effects of BAPTA and other secretagogues, as well as SP-A, on $[Ca^{2+}]_i$

Changes in Ca^{2+} levels within ER stores were measured from the ratio of fura-2ff fluorescence induced at 340 nm (fura-2ff bound to Ca^{2+}) to that induced at 380 nm (unbound), as described in the Materials and methods section. In these representative tracings, cells were preloaded with fura-2ff, followed by $MnCl_2$ added at zero time [25]. Additions were made at the indicated times: (a) BAPTA-AM; (b) Ionomycin; (c) PMA; (d) SP-A; (e) mock.

secretagogues stimulate surfactant secretion via different pathways, surfactant secretion after treatment with both simultaneously might exceed that elicited by either alone. PMA induces surfactant secretion without increasing $[Ca^{2+}]_i$, even in Ca^{2+} -depleted cells. Its secretagogue activity could thus reflect a different signalling pathway or, less probably, action at a different point in the same pathway. Combinations of Ca^{2+} -releasing secretagogues and PMA were tested for secretagogue activity. Adding two Ca^{2+} -releasing secretagogues together did not increase surfactant secretion beyond that seen with either individually (Figure 5). However, when PMA was added to a Ca^{2+} -releasing secretagogue, the observed secretagogue activities were more or less additive.

Cell viability was measured in all studies. Some combinations of secretagogues (PMA plus BAPTA, and BAPTA plus TG)

were toxic to type II cells (at least at the concentrations tested), so additivity of these secretagogue activities could not be assessed.

Effect of BAPTA on $[Ca^{2+}]_i$

BAPTA, a Ca^{2+} chelator, required Ca^{2+} to activate surfactant secretion but should not alter $[Ca^{2+}]_i$. To ascertain this, we followed $[Ca^{2+}]_i$ in type II cells that were loaded with fura-2 and then exposed to BAPTA-AM. As measured by a decrease in the ratio of fura-2 excitation at 340 nm compared with that at 380 nm, BAPTA-AM caused $[Ca^{2+}]_i$ to decrease somewhat, compared with mock-treated cells (Figure 6). Therefore BAPTA-AM is a Ca^{2+} -dependent secretagogue that does not increase $[Ca^{2+}]_i$.

Alterations in luminal Ca²⁺ concentration in response to Ca²⁺-releasing secretagogues, PMA, BAPTA and SP-A

To explain these results we hypothesized that decreases in [Ca²⁺]_i, rather than increases in [Ca²⁺]_i, were the critical regulator of surfactant secretion by all secretagogues except PMA. This theory predicts that BAPTA-AM, and other Ca²⁺-dependent secretagogues, would decrease [Ca²⁺]_i but that PMA would not. Thus we loaded type II cells with fura-2ff. This fluorophore has a much lower affinity for Ca²⁺, in the micromolar range, than does fura-2 and so can be used to assess changes in [Ca²⁺]_i. We also treated the type II cells with Mn²⁺, which binds cytoplasmic fura-2ff and prevents its binding to Ca²⁺ [27,29,30]. Induced changes in the ratio of fura-2ff excitation at 340 nm to that at 380 nm were the measure of luminal free Ca²⁺ concentration. As predicted, BAPTA decreased [Ca²⁺]_i, as did Io (Figure 7). PMA had no effect.

Effect of SP-A on luminal Ca²⁺ concentration

To explain the inhibition by SP-A of both secretagogue-induced Ca²⁺ release and secretagogue-induced surfactant secretion, we hypothesized further that SP-A might increase the stores' avidity for Ca²⁺, increasing Ca²⁺ uptake and rendering luminal Ca²⁺ less susceptible to release. SP-A alone should then increase [Ca²⁺]_i. This was tested as described above, in type II cells loaded with fura-2ff and treated with Mn²⁺ as above. SP-A caused increased [Ca²⁺]_i in type II cells (Figure 7).

DISCUSSION

The results presented here support the centrality of Ca²⁺ signalling in surfactant secretion and suggest an important role for [Ca²⁺]_i in that signalling. We propose that the ability of BAPTA to stimulate surfactant secretion is a key to understanding both signalling by Ca²⁺-releasing secretagogues and its inhibition by SP-A.

We found that the secretagogue activities of ATP, Io, and TG were all inhibited by depleting cytosol Ca²⁺ and were then restored by the replenishment of Ca²⁺. PMA-stimulated secretion, which seems to circumvent Ca²⁺ signalling in type II cells, was unaffected by these manoeuvres.

TG-induced secretion was less completely inhibited by depleting cytosol Ca²⁺ than was secretion induced by other Ca²⁺-releasing secretagogues. The reasons for this are unknown. TG might for example access a store of Ca²⁺ (such as high-affinity binding sites) not easily depleted by overnight culture without Ca²⁺ and not accessible to other secretagogues.

Ca²⁺ influx is not a major participant in signalling surfactant secretion. When Ca²⁺ was removed from the medium at assay time, BAPTA- or Io-induced secretion was unchanged. Small decreases in TG- and PMA-induced secretion seen in these studies were of borderline statistical significance ($P = 0.03$) and their cause is not clear.

Ca²⁺ starvation did not make surfactant totally unavailable for secretion, because PMA-induced secretion was largely unaffected. PL synthesis was unaffected: incorporation of choline and glycerol precursors into PLs was similar in Ca²⁺-starved and control cells.

In this regard the ability of a cation chelator, BAPTA, to stimulate type II cells to secrete surfactant is illuminating. The cation involved in BAPTA-induced secretion was Ca²⁺. TPEN, which chelates transition metal ions such as iron or Zn²⁺ well, and Ca²⁺ poorly [31,32], did not stimulate surfactant secretion. More importantly, the secretagogue activity of BAPTA was eliminated by depleting Ca²⁺ and restored by replacing it. Another

group reported using BAPTA in type II cell cultures but at 5 μ M [33]. In our dose-response studies this concentration was below the threshold for BAPTA to stimulate surfactant secretion.

The ability of BAPTA to stimulate secretion did not involve depleting the total cellular pool of Ca²⁺, as Ca²⁺ deprivation overnight did not itself elicit large-scale surfactant secretion. There are several possible explanations for these findings. As in other systems [34,35], Ca²⁺ might be needed further downstream for surfactant secretion, independently of signalling. BAPTA buffering cellular Ca²⁺ would not block such a Ca²⁺-requiring step, whereas depleting cellular Ca²⁺ would. Ca²⁺ might also help to maintain the structure of ER stores, and its depletion might distort them and render them less effective in transducing secretion-activating signals [36].

From the perspective of type II cell surfactant secretion, BAPTA-AM acts like a Ca²⁺-releasing secretagogue. It causes surfactant secretion in a dose-dependent manner, is inhibited by depleting Ca²⁺, does not require Ca²⁺ influx, and is inhibited by SP-A. However, unlike Ca²⁺-releasing secretagogues, BAPTA does not increase [Ca²⁺]_i. Therefore Ca²⁺ signalling in type II cell PL secretion is unlikely to be related to increased [Ca²⁺]_i.

Ca²⁺ in ER stores is in equilibrium between free and protein-bound states. If the free Ca²⁺ in stores is chelated or released, the equilibrium shifts, decreasing the amount of bound Ca²⁺. BAPTA in its esterified form should easily access ER stores [30,37], where it can be de-esterified. The high affinity for Ca²⁺ (160 nM) should compete effectively with Ca²⁺-binding proteins there, and decrease bound Ca²⁺. To explain our results, we suggest that Ca²⁺ signalling activates surfactant secretion by altering [Ca²⁺]_i, not by increasing [Ca²⁺]_i.

To test this hypothesis, we measured [Ca²⁺]_i directly, applying a combination of fura-2ff and Mn²⁺ quenching of cytosol fura-2ff fluorescence. This approach has been used previously as a method of measuring [Ca²⁺]_i [25,27,29,30]. This analysis highlighted the fact that BAPTA and Ca²⁺-releasing secretagogues all decreased [Ca²⁺]_i. PMA, which does not release Ca²⁺ from stores, had no effect on [Ca²⁺]_i. These findings are best explained by postulating that surfactant secretion as elicited by Ca²⁺-active secretagogues is triggered by a decrease in Ca²⁺ concentration in the ER store: free Ca²⁺, [Ca²⁺]_i and/or bound Ca²⁺. Alternatively, the process of Ca²⁺ release, rather than the consequent decrease in [Ca²⁺]_i, may trigger secretion.

ER Ca²⁺ stores would then regulate, directly or indirectly, lamellar body movement to the cell surface for extrusion. For example, Ca²⁺ release might alter the conformation of a Ca²⁺-binding protein that spans the ER membrane, such as Ins(1,4,5)P₃R [16]. In changing configuration, Ins(1,4,5)P₃R might initiate a signal to move lamellar bodies to the cell surface.

Our observations with SP-A both support the importance of [Ca²⁺]_i in signalling surfactant secretion and suggest a method by which SP-A inhibits secretagogue-induced surfactant secretion. SP-A binds SPAR, inhibiting surfactant secretion [6,12,29,38]. It also modulates the secretagogue-induced Ca²⁺ transients that we and others have reported [4,7,26,27]. It is noteworthy that Io- and TG-induced increases in [Ca²⁺]_i were also partly inhibited by SP-A (and restored if SP-A-SPAR interaction is blocked with anti-SPAR antibody [6]), because Io and TG act on ER stores directly. The ability of SP-A to decrease secretagogue-induced increases in [Ca²⁺]_i suggests that SP-A alters the availability of Ca²⁺ for release.

The fact that SP-A increased [Ca²⁺]_i in a high percentage of type II cells therefore both helps to substantiate the proposed mechanism for the activity of Ca²⁺-releasing secretagogues and suggests that the activity of SP-A in inhibiting their action is by increasing [Ca²⁺]_i. The mechanism(s) by which SP-A does this is

unclear. SP-A might for example increase the avidity with which stored Ca^{2+} is bound or alternatively it might increase the activity of the Ca^{2+} pump across the ER store membrane.

Our studies with PMA underscore the suggested relationship between the ability of a secretagogue to elicit Ca^{2+} transients and its dependence on Ca^{2+} for signalling secretion. PMA secretagogue activity circumvents Ca^{2+} signalling [7]. PMA also did not alter $[\text{Ca}^{2+}]_i$. The inhibition of PMA-induced secretion by SP-A probably involves mechanisms other than SP-A-induced increases in $[\text{Ca}^{2+}]_i$.

Combining Ca^{2+} -releasing secretagogues did not increase secretion: only PMA augmented secretion elicited by individual Ca^{2+} -releasing secretagogues. Combinations including BAPTA plus TG and BAPTA plus PMA killed the cells. Apoptosis associated with TG has been reported previously [39,40]. Nevertheless, these results suggest that PMA activates secretion either via a different signalling mechanism or, less probably, distal to Ca^{2+} release in the pathway activated by Ca^{2+} -releasing secretagogues.

The extent to which the current results apply to other systems of Ca^{2+} -related secretion is not clear. In some experimental systems, BAPTA has no clear effect on secretory activity [15], whereas in many experimental systems the chelation of Ca^{2+} by BAPTA inhibits cellular secretion [41,42]. In cell types other than type II cells, BAPTA inhibited the secretagogue activities of the same compounds whose secretagogue activities it mimicked here [43–45]. Several studies describe BAPTA and (for example) Io as having parallel effects in cellular secretion [44,45]. In none of these systems, however, did BAPTA activate secretion.

The present paper focuses attention on Ca^{2+} in stores, and away from $[\text{Ca}^{2+}]_i$, as a key effector in signalling type II pneumocyte surfactant secretion. The extent to which similar mechanisms might operate in other Ca^{2+} -dependent cell activation pathways in other cell types is not known. These findings might also have clinical ramifications, which remain to be explored.

Note added in proof (received 4 October 1999)

Since the original submission of this manuscript, another report has appeared suggesting that depletion of Ca^{2+} from ER stores may activate signalling [46].

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