Ca^{2+} dependency of 'Ca^{2+}-independent' exocytosis in SPOC1 airway goblet cells

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SPOC1 airway goblet cells secrete mucin in response to $P2Y_2$ receptor agonists and to secretagogues, phorbol 12-myristate 13-acetate (PMA) and ionomycin, which mobilize elements of the phospholipase C pathway, PKC and Ca^{2+}, respectively. Previous studies demonstrated that mucin secretion from SLO-permeabilized, EGTA-buffered SPOC1 cells was stimulated by PMA at low Ca^{2+} levels ($< 0.1\ \mu$M), consistent with the notion that regulated exocytosis may occur by Ca^{2+}-independent pathways. We tested the alternative hypothesis that PMA-induced mucin secretion is, in fact, a Ca^{2+}-dependent process under the conditions of low bulk Ca^{2+}, one that is permitted in the typical SLO-permeabilized cell model by the slow binding kinetics of EGTA. Both IP_3 and elevated bulk Ca^{2+} activated mucin secretion in SPOC1 cells buffered by EGTA, suggesting that IP_3 generates a local Ca^{2+} gradient in the vicinity of the secretory granules to the degree necessary to trigger exocytosis. BAPTA, which binds Ca^{2+} approximately 100-fold faster than EGTA, diminished IP_3-induced mucin release over a range of concentrations by $\geq 69\%$, yet maintained an essentially normal mucin secretory response to elevated bulk Ca^{2+} in permeabilized SPOC1 cells. BAPTA also diminished the mucin secretory response of permeabilized cells to PMA, relative to the EGTA-buffered control: at PMA below 30 nM, BAPTA abolished the secretory response, and at higher concentrations it was reduced significantly relative to the EGTA-buffered controls. PMA-induced secretion in EGTA was insensitive to heparin. These results suggest that Ca^{2+} is released locally during PMA-induced exocytosis, by an IP_3-independent mechanism.

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Airway goblet cells and submucosal glands secrete the high molecular weight glycoconjugate mucin, the viscoelastic component of mucus. In healthy lungs, mucus plays a principal role in mucociliary clearance (Knowles & Boucher, 2002). However, in the airway obstructive diseases asthma, chronic bronchitis, bronchiectasis, and cystic fibrosis, mucus/mucin hypersecretion is a hallmark characteristic that arises from inflammation-induced goblet cell and submucosal gland hyper- and metaplasia (reviewed by Rogers, 2003). Despite the central role of mucin secretion in health and its importance in airway disease, the regulation of mucin granule exocytosis is poorly understood beyond the level of receptor activation and cellular messenger generation.

SPOC1 cells, derived from rat tracheal epithelium (Randell *et al.* 1996), secrete mucin in response to purinergic stimulation by ATP or UTP activation of $P2Y_2$ receptors (Abdullah *et al.* 1996), in agreement with studies on goblet cells in primary cultures from hamster trachea (Kim & Lee, 1991), canine tracheal epithelial explants (Davis *et al.* 1992), and human bronchial epithelial cells (Chen *et al.* 2001; Conway *et al.* 2003). $P2Y_2$ receptors typically couple to PLC (Harden *et al.* 1995), and mobilization of intracellular Ca^{2+} by ionomycin and activation of PKC by the DAG mimic, PMA, stimulate mucin secretion in SPOC1 cells (Abdullah *et al.* 1997; Abdullah *et al.* 2003). Much of the SPOC1 cell data on the regulation of mucin secretion by Ca^{2+} and PKC are consistent with the notion that these effectors function independently. Chief among these are the findings that ionomycin and PMA effects are fully additive at maximal concentrations (Abdullah *et al.* 1997), that the agonist-responsive isoform of PKC, nPKCδ, is a member of the novel, Ca^{2+}-independent PKC subfamily (Abdullah *et al.* 2003), and that in permeabilized, EGTA-buffered cells PMA stimulates mucin secretion at 10 nM free Ca^{2+}, an order of magnitude below generally accepted basal levels of intracellular Ca^{2+} (Scott *et al.* 1998). Ca^{2+}-independent,

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regulated exocytosis has also been proposed for other secretory cells: (i) non-hydrolysable analogues of GTP stimulate degranulation of permeabilized, EGTA-buffered mast cells (Gomperts *et al.* 1986), and in mast cells dialysed with EGTA buffer by a whole-cell patch pipette, exocytotic events sensed by changes in membrane capacitance failed to correlate with Ca^{2+} transients observed by fura-2 fluorescence (Neher & Almers, 1986); (ii) in gonadotropes of the anterior pituitary, PMA stimulates luteinizing hormone (LH) secretion in the absence of observable changes in Ca^{2+} (Betz *et al.* 1998); and (iii) in pancreatic duct epithelial cells, exocytosis is stimulated by agents which elevate cAMP, without observable changes in Ca^{2+} (Koh *et al.* 2000). Hille *et al.* (1999) have postulated that such data indicate a Ca^{2+}-independent regulation of exocytotic secretion by protein kinases such as PKC and PKA.

Contrary to the notion of a mechanism for regulated exocytosis that is independent of Ca^{2+}, there is a strong argument that the process is strictly Ca^{2+} dependent. Ca^{2+} plays important roles in the regulation of exocytosis, beginning with cortical actin filament disassembly (Trifaro *et al.* 2000), granule docking to the plasma membrane (Martin, 2002), and fusion of the granule membrane to the plasma membrane during pore formation (Gerber & Sudhof, 2002). In fact, the postulated trigger for regulated exocytosis is synaptotagmin, an obligate accessory protein to the exocytotic SNARE complex. Though the number of studies is at present small, only the Ca^{2+}-dependent synaptotagmin isoforms have been associated with regulated exocytosis (Chapman, 2002; Sudhof, 2002).

Interestingly, experiments yielding the most direct evidence favouring Ca^{2+}-independent exocytosis are typically buffered by EGTA. Because this Ca^{2+} buffer has relatively slow binding kinetics (Tsien, 1980), one explanation of apparent Ca^{2+}-independent exocytosis is that EGTA permits the generation of local Ca^{2+} gradients. Hence, we used IP_3 and other manoeuvres to test for the existence of Ca^{2+} gradients in permeabilized, EGTA-buffered cells, and using BAPTA as a probe we tested the hypothesis that regulated SPOC1 cell mucin secretion is, in fact, Ca^{2+} dependent.

Methods

Materials

Dulbecco's modified Eagle's medium–Ham's nutrient mixture F12 (DMEM–F12) was obtained from Gibco BRL (Gaithersburg, MD, USA) and the supplements from Collaborative Research (Bedford, MD, USA). Bisindolylmaleimide II (BIMII), calphostin C, D-*myo*-inositol 1,4,5-trisphosphate (IP_3; trilithium salt), heparin (sodium salt; low molecular weight), and phorbol-12-myristate-13-acetate (PMA) were purchased from Calbiochem (La Jolla, CA, USA). Ethylene gylcol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was purchased from Sigma (St Louis, MO, USA); 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′*N*′-tetraacetic acid (BAPTA; tetrapotassium salt) and TO-PRO were purchased from Molecular Probes (Eugene, OR, USA). Streptolysin-O (SLO) was purchased from Corgenix (Peterborough, UK).

SPOC1 cell culture

SPOC1 cells were seeded in 48-well cluster plates (Costar, Cambridge, MA, USA) at 25 000 cells per well (Scott *et al.* 1998) and maintained in rat tracheal epithelial cell medium (Randell *et al.* 1996), as previously described. The cells, passage 7–15, were used for experiments 18–22 days post-confluence.

SLO permeabilization and mucin release

SPOC1 cells were equilibrated before each experiment, as follows, using a Finnpipette multistepper, multichannel pipetter (Needham Heights, MA) and a custom fabricated, multichannel aspirator to quickly remove and replace all solutions from 48-well culture plates. Cells were placed in a 37°C water bath, washed twice with DMEM–F12 (200 μl well^{-1}), and incubated for 30 min; this procedure was repeated three times. Cells were washed twice with phosphate buffered saline (PBS) and then with intracellular buffer (Buf_i, composition given below). Immediately following the washes, SPOC1 cells were permeabilized with the bacterial toxin streptolysin-O (SLO) at a final concentration of 1 U ml^{-1} in Buf_i for 30 s (150 μl well^{-1}), as previously described (Scott *et al.* 1998). Following the permeabilization, cells were washed once in Buf_i and incubated with the appropriate solution (200 μl well^{-1}; see Results) for 15 min, at which time the samples were collected for the determination of mucin content. TO-PRO, a membrane-impermeant, DNA-binding fluorescent dye, was used to confirm cell permeability, as previously described (Scott *et al.* 1998). Notably, TO-PRO and BAPTA are similar in size (relative molecular mass 645 *versus* 628.8) and EGTA is approximately 50% smaller (relative molecular mass 380.4), making the dye a relevant marker of small molecule permeation. In all of the studies reported below, TO-PRO fluorescence was observed to be uniformly distributed throughout SPOC1 cell cytoplasm indicating that the cells were well permeabilized.

Mucin enzyme-linked lectin assay

Samples collected from each well of the 48-well culture plate were assessed for mucin content using an enzyme-linked lectin assay (ELLA), previously described

(Abdullah *et al.* 1996). Briefly, samples were diluted (100 μl final volume) and bound to 96-well high-binding microtitre plates (Costar), incubated overnight at 4°C or 37°C for 2 h, washed with PBST (0.05% Tween 20 in PBS), and incubated with 2.5 μg ml^{-1} peroxidase-labelled soybean lectin for 1 h at 37°C. The plates were then washed and developed with *O*-phenylenediamine in a citrate phosphate buffer (0.0175 M, 0.01% hydrogen peroxide, pH 5.0). H_2SO_4 (4 M) was used to stop the reaction, and the plates were analysed using optical density at 490 nm (Dynatech microtitre plate reader, model MR5000; Chantilly, VA, USA). Known amounts of purified SPOC1 mucin were used to generate standard curves, on each plate, allowing the results to be expressed as nanograms mucin released per culture.

Intracellular Ca^{2+} buffer

EGTA stock solutions (50 mM) were prepared as previously described (Gomperts & Tatham, 1992; Scott *et al.* 1998) after first titrating the EGTA with $CaCl_2.2H_2O$ to determine true buffer concentrations (Miller & Smith, 1984). BAPTA stock solutions (25 mM) were calculated directly, with allowance made for impurities as determined by HPLC (Molecular Probes, OR, USA). These stocks were stored at −20°C. Free Ca^{2+} activities were computed with the aid of the computer program Chelator (Schoenmakers *et al.* 1992) and CaEGTA or CaBAPTA were used to adjust the final free Ca^{2+} levels. Except where stated, both Ca^{2+} buffers were used at a final concentration of 3 mM. Intracellular buffer (Buf_i) contained 130 mM potassium glutamate, 20 mM Pipes, 1 mM MgATP and $MgCl_2$, and 3 mM EGTA or BAPTA (pH 6.8). Free Ca^{2+} in Buf_i was set to 0.1 μM, or, where stated in Results, was varied from 0.01 to 10 μM.

Ca^{2+} buffer simulations

Simulations were generated to compare the effectiveness of BAPTA and EGTA to buffer Ca^{2+} over time and space. For the temporal simulation (Fig. 2*A*), the free Ca^{2+} concentration was calculated from a consideration of the rate of change of bound Ca^{2+} following a step addition of EGTA or BAPTA to a solution containing 10 μM free Ca^{2+}. The rate of change of bound Ca^{2+}, D, is:

$$\frac{dD}{dt} = k_+ D - k_- D,$$

with association rate constant k_+, dissociation rate constant k_-, buffer concentration B, Ca^{2+} concentration C, complex concentration BC, and time t. This equation can be put into the form:

$$\frac{dD}{dt} = a_0 + a_1 D + a_2 D^2,$$

Table 1. Parameters used in temporal and spatial Ca^{2+} gradient simulations

Ca^{2+} total influx (ions s^{-1})[a]	10^6
Ca^{2+} diffusion coefficient (μm^2 s^{-1})[a]	220
Total buffer concentration (mM)[c]	3
Bulk Ca^{2+} concentration (μM)[c]	0.1

	EGTA	BAPTA
Buffer diffusion coefficient (μm^2 s^{-1})[b]	113	95
Thermodynamic dissociation constant (μM)[b]	0.20	0.17
Association rate constant (μM^{-1} s^{-1})[b]	1.5	600

[a]Bauer (2001), [b]Smith *et al.* (2001). [c]Parameters from permeabilized SPOC1 cell experiments.

which has a solution,

$$D(t) = \frac{1}{2a_2}\left(\gamma \frac{a_1 - \gamma \tanh(\gamma t/2)}{\gamma - a_1 \tanh(\gamma t/2)} - a_1\right), \quad (1)$$

where, at $D(0) = 0$,

$$\gamma = \sqrt{{a_1}^2 - 4a_0 a_2}.$$

The spatial simulation (Fig. 2*B*) compares the steady state free Ca^{2+} concentration profiles in the presence of BAPTA, EGTA, or no buffer on one side of a planar boundary with a point Ca^{2+} source. The profile can be modelled using an approximation to the reaction–diffusion problem first described by Neher (1986) and now known as the excess buffer approximation (for review, see Stern, 1992; Bauer, 2001; Smith *et al.* 2001). The approximation is valid under conditions of high buffer concentration, low bound buffer to total buffer ratios, and rapid buffer diffusion. In this simulation, Bauer's excess buffer approximation for the case of one buffer was used, with the parameters listed in Table 1, to calculate steady state free Ca^{2+} concentration as a function of distance from a pore (eqn (9) in, Bauer, 2001). The calculated bound to total ratios for BAPTA and EGTA at the channel were 0.067 and 0.0029, respectively (eqn (12b) in, Bauer, 2001).

Statistical analysis

Data collected from the ELLA were normalized to mucin secretion at 0.01 μM Ca^{2+} under control conditions. Data are presented as the mean ± S.E.M., for a specified number of SPOC1 cell cultures, each culture in a given experiment originating from a different passage. ANOVA and Student's t test were used to determine statistical significance between data sets. * indicates P-values < 0.05.

Results

IP_3-induced Ca^{2+} gradients

To test whether significant Ca^{2+} gradients can be generated in SLO-permeabilized, EGTA-buffered SPOC1 cells we used exogenous IP_3 to release Ca^{2+} from internal stores. Cells grown on 48-well culture plates were exposed to IP_3 (0.03–30 μM) in 0.1 μM Ca^{2+} EGTA buffer on one half of the plate. As controls, cells on the other half of the plate were exposed to a series of Ca^{2+} activities (0.01–10 μM) buffered by EGTA. Mucin secretion induced by increases in bulk Ca^{2+} was enhanced in a concentration-dependent manner at activities $> 1\ \mu$M (Fig. 1*A*), consistent with previous studies in EGTA-buffered SPOC1 cells and many other secretory cells (Scott *et al.* 1998; and references therein). IP_3 also caused a concentration-dependent increase in mucin secretion with a maximum response at 0.3 μM, a value close to its reported EC_{50} (Wojcikiewicz & Luo, 1998). For reasons that are not clear, IP_3 concentrations $> 0.3\ \mu$M were less effective in stimulating mucin secretion in these cells. Notably, the maximal mucin secretory response to IP_3 (0.3 μM) was approximately equal to that induced by 10 μM bulk Ca^{2+}, suggesting that IP_3 induces local Ca^{2+} release either in the vicinity of, or from, mucin secretory granules in EGTA-buffered cells generating a local gradient with a maximal effective concentration of $\sim$10 μM. Heparin, an IP_3 receptor inhibitor, blocked the mucin secretory response to IP_3(Fig. 1*B*), suggesting that the IP_3 effect to release Ca^{2+} is specific.

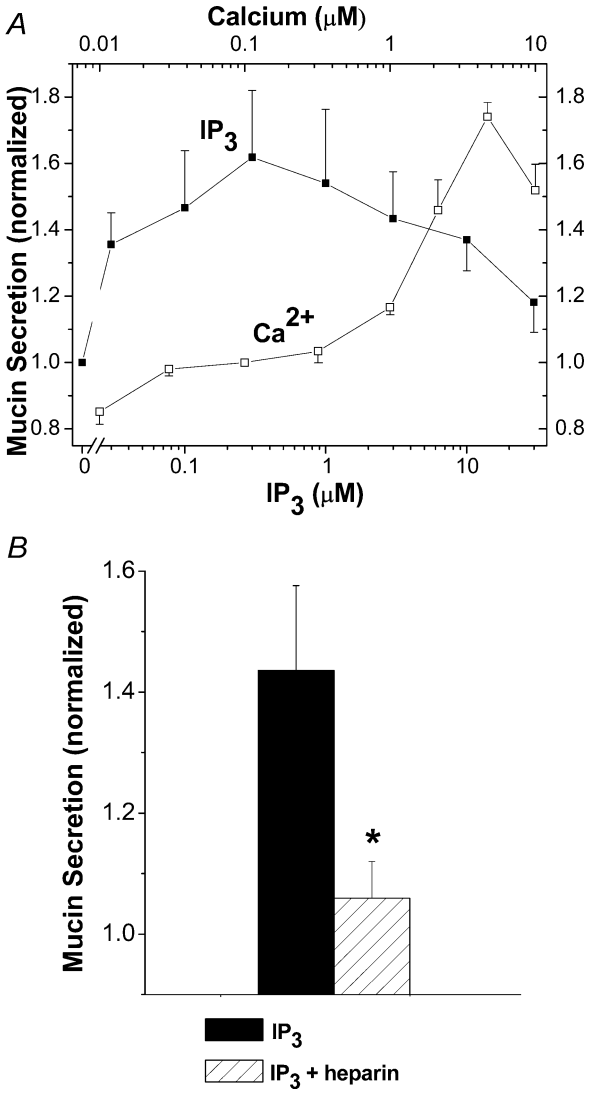

Figure 1. Effects of IP_3 and bulk Ca^{2+} on SLO-permeabilized, EGTA-buffered SPOC1 cells
A, cells grown on one half of a 48-well plate were incubated with IP_3 (0.03–30 μM; 0.1 μM Ca^{2+}), whereas the other half received different concentrations of Ca^{2+} (0.01–10 μM; no IP_3). Following permeabilization in this and all other experiments, each treatment was applied to triplicate wells and the mucins released were collected and assessed after a 15 min incubation using a lectin-linked, microtitre plate binding assay. In each case, mucin release was normalized to the secretions at 0.1 μM Ca^{2+} and is represented as the mean ± S.E.M. ($n = 6$ SPOC1 passages). *B*, inhibition of IP_3-induced mucin release by heparin. Cells were exposed to 0.3 μM IP_3 ± 200 μg ml^{-1} heparin ($n = 6$ SPOC1 passages).

Buffer kinetics: relative effects on free Ca^{2+} and mucin secretion

The two Ca^{2+} chelators commonly used in biomedical experiments, EGTA and BAPTA, have similar buffer affinities, in contrast to vastly different binding kinetics (see Table 1). To determine whether the faster binding properties of BAPTA might be used to our advantage, we first simulated the time course of Ca^{2+} binding by the two buffers using starting conditions that mimicked those likely to occur in permeabilized SPOC1 cells under our experimental conditions, i.e. 10 μM free Ca^{2+} and 3 mM buffer. The simulations indicate that addition of BAPTA, with instantaneous mixing, decreases free Ca^{2+} to vanishingly low activities in $\sim$2.5 μs, a time during which EGTA addition has no significant effect (Fig. 2*A*). As indicated in the figure, EGTA binds only $\sim$50% of the total free Ca^{2+} over an extended period of time (150 μs). Therefore, short-lived Ca^{2+} transients such as those that occur in nerve terminals will be effectively buffered by BAPTA, but not EGTA (e.g. see Adler *et al.* 1991).

Ca^{2+} release events associated with G protein-coupled receptor (GPCR) signalling, by comparison to those at synaptic terminals, generally have much longer durations (tens of seconds). In this case, steady-state Ca^{2+} gradients are likely to be established in the vicinity of Ca^{2+} release sites. Because small, mobile Ca^{2+} buffers will determine the Ca^{2+} concentration profile centred on such sites (see Bauer, 2001; Smith *et al.* 2001), we simulated Ca^{2+} gradients generated in the vicinity of a channel opening in the presence of EGTA or BAPTA.

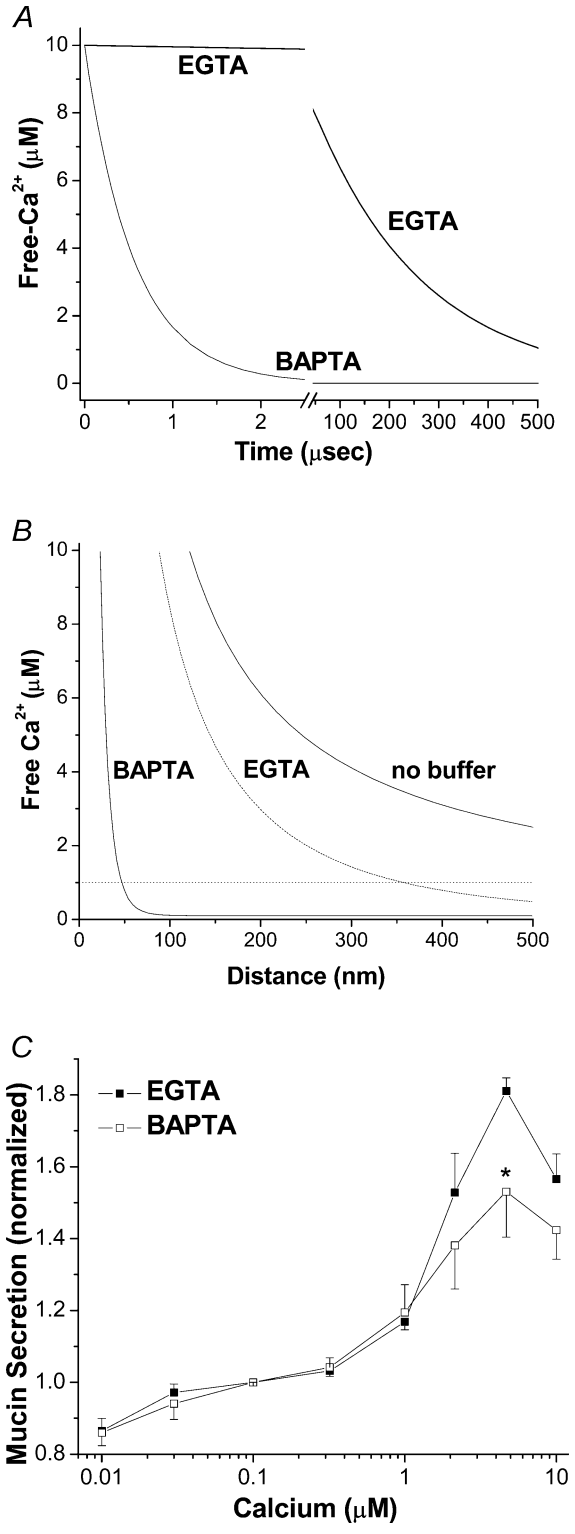

Figure 2. Binding kinetics of EGTA and BAPTA and their relative effectiveness in Ca^{2+}-activated mucin secretion
A, temporal simulation using eqn (1), illustrating the Ca^{2+} binding kinetics of EGTA and BAPTA and their effects on free Ca^{2+} over 500 μs following the step addition of 3 mM buffer to a solution of 10 μM free-Ca^{2+}. *B*, spatial simulation of steady state free Ca^{2+} concentration plotted as a function of the distance along a radial extending from a channel opening in a planar membrane. *C*, comparison of mucin secretion activated in SPOC1 cells by bulk Ca^{2+} (0.01–10 μM) when buffered by EGTA or BAPTA ($n = 6$ SPOC1 passages).

Under our experimental conditions, BAPTA is predicted to buffer Ca^{2+} much closer to the channel than would EGTA (Fig. 2*B*). At a distance < 50 nm from the mouth of an open channel, with a flux of 10^6 ions s^{-1}, BAPTA will buffer Ca^{2+} to levels below 1 μM, whereas a gradient of similar magnitude in EGTA will extend > 350 nm. Hence, BAPTA is an excellent probe for revealing local Ca^{2+} gradients as well as Ca^{2+} transients.

As an initial control for potential buffer effects on mucin release, we tested the effects of elevated Ca^{2+} on mucin secretion from SPOC1 cells permeabilized into EGTA- or BAPTA-based buffers. As shown in Fig. 2*C*, mucin secretion activated by bulk Ca^{2+} was similar in both EGTA- and BAPTA-buffered conditions, with the exception of 3 μM Ca^{2+} where, though mucin secretion was still elevated in BAPTA-buffered cells, it was diminished relative to EGTA.

The findings in Fig. 1 indicate that IP_3 releases a significant amount of Ca^{2+} near sites of mucin granule exocytosis at the apical membrane in EGTA-buffered, permeabilized cells. In experiments conducted with cells of the same passage, we tested whether BAPTA, in the place of EGTA, would diminish mucin secretion elicited by IP_3. As shown in Fig. 3, mucin secretion in BAPTA-buffered cells was inhibited by 69–97% at all IP_3 concentrations, relative to EGTA.

Lack of buffer effects

Previous studies suggested that BAPTA might have effects independent of Ca^{2+} buffering. For instance, in studies with purified IP_3 receptors from the cerebellum, BAPTA, EDTA and EGTA were shown to inhibit IP_3 binding. The inhibition was competitive between IP_3 and the free forms of the buffer (Richardson & Taylor, 1993). Similarly, free-BAPTA was implicated in an inactivation of PKC in liver macrophages (Dieter *et al.* 1993). Because BAPTA suppressed mucin secretion slightly, but significantly, at high Ca^{2+} in permeabilized SPOC1 cells (Fig. 2*C*), we tested for possible direct effects of EGTA and BAPTA on mucin secretion. First, to test whether PKC inactivation might explain the apparent inhibition by BAPTA (Fig. 2*C*) we used calphostin C and BIMII, in combination, to inhibit PKC (Fig. 4*A*) during stimulation by high Ca^{2+}. The data indicate no effect of the inhibitors at 3 μM Ca^{2+} in EGTA- or BAPTA-buffered SPOC1 cells. Mucin secretion was stimulated, though diminished, with BATPA buffering, relative to EGTA, consistent with data of Fig. 2*C*. Second, as a more general test of Ca^{2+}-independent effects of chelator we measured IP_3-induced mucin release at a constant Ca^{2+}

activity of 0.1 μM, over a broad range of EGTA and BAPTA concentrations (0.03–10 mM; Fig. 4*B*). The results indicate that IP_3 elicited mucin secretion independent of EGTA concentration, and that BAPTA generally abolished the stimulatory effects of IP_3, again, independent of buffer concentration. Note that in this experiment (cf. Fig. 3), the only point where BAPTA failed to abolish IP_3-induced mucin secretion was at 10 μM, a concentration at which Ca^{2+} buffering by BAPTA is likely to be ineffective. Hence, from these studies we conclude that there were no apparent direct effects of either chelator on the secretory response. Importantly, the lack of buffer concentration effects on IP_3-stimulated mucin release in EGTA, or its inhibition by BAPTA, also makes it highly unlikely that the differences observed between the two buffers can be ascribed to differences in permeation of the buffer.

Ca^{2+} dependency of PMA-induced mucin secretion

Because BAPTA suppressed IP_3-induced Ca^{2+} gradients, we used it to probe whether the stimulatory effects of PMA on mucin secretion are also Ca^{2+} dependent. Similar to our previous results (Scott *et al.* 1998), a concentration of PMA eliciting maximal effects (300 nM) stimulated mucin secretion from EGTA-buffered SPOC1 cells at all Ca^{2+} activities tested, including those clearly sub-basal to normal resting Ca^{2+} (Fig. 5). PMA increased mucin release by ~100% at low Ca^{2+} activities and up to ~350% at 3 μM Ca^{2+}. Using BAPTA as the buffer, however, reduced PMA-stimulated mucin secretion by 32–58%, relative to EGTA-buffered cells. Experiments using 10 mM EGTA and BAPTA instead of 3 mM yielded similar results (data not shown).

Recent studies from our laboratory identifying the $P2Y_2$ agonist- and PMA-sensitive isoforms of PKC in SPOC1 cells suggested that the effects of PMA to stimulate mucin secretion at concentrations > 30 nM are independent of PKC (Abdullah *et al.* 2003). Hence, we compared the effects of EGTA and BAPTA buffering on concentration-dependent PMA stimulation of mucin secretion from SLO-permeabilized SPOC1 cells. Cells on 48-well cluster plates were buffered (0.1 μM free Ca^{2+}) by EGTA on one half of a plate and by BAPTA on the other half, and incubated with 0.3–300 nM PMA. As shown in Fig. 6*A*, there was a biphasic response of EGTA-buffered SPOC1 cells to PMA. Mucin secretion was stimulated significantly over baseline between 1 and 30 nM PMA, the range within

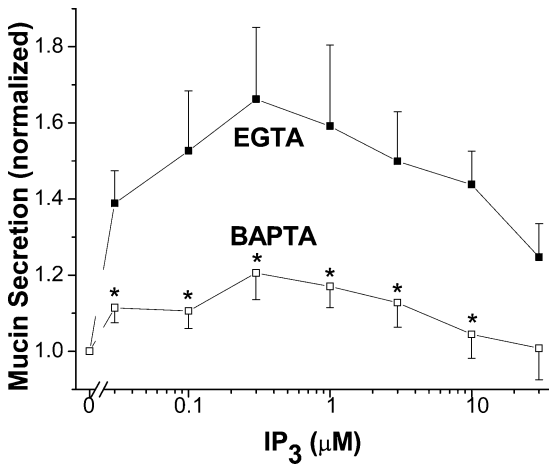

Figure 3. Effects of rapid Ca^{+} buffering on IP_3-induced mucin secretion from SPOC1 cells
SLO-permeabilized cells were exposed to IP_3 (0.03–30 μM), buffered by EGTA or BAPTA (0.1 μM Ca^{2+}). The EGTA data duplicate those in Fig. 1; the experiments were conducted at the same time, on paired 48-well plates. Two-way ANOVA indicates a significant effect of BAPTA ($n = 6$ SPOC1 passages).

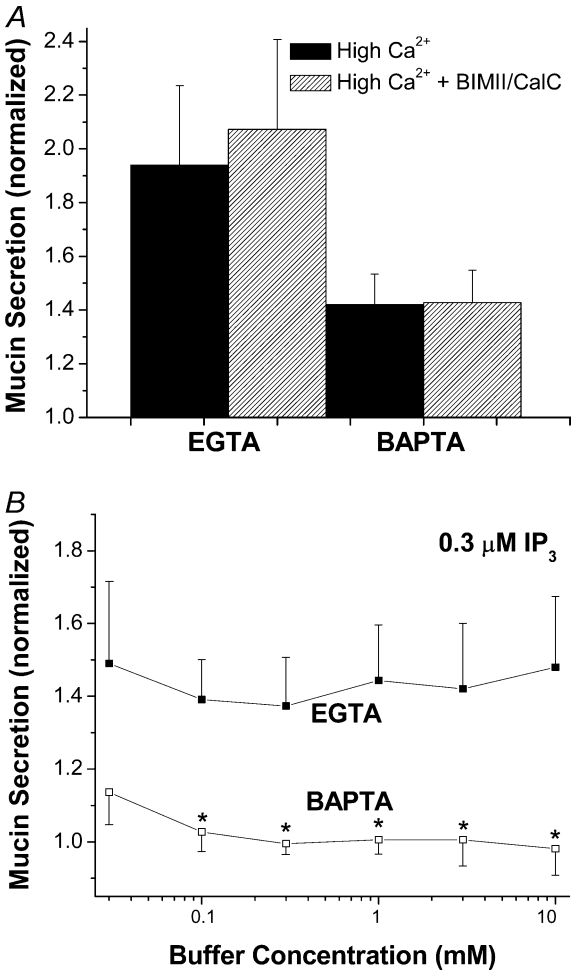

Figure 4. Lack of buffer effects on mucin secretion in SLO-permeabilized SPOC1 cells
A, cells were exposed to high Ca^{2+} (3 μM), buffered by EGTA or BAPTA (0.1 μM Ca^{2+}), ± the PKC inhibitors BIMII (1 μM) and calphostin C (1 μM; $n = 4$ SPOC1 passages). *B*, cells were exposed to IP_3 (0.3 μM) and buffered by either EGTA or BAPTA (0.03–10 mM) at a constant 0.1 μM Ca^{2+} ($n = 4$ SPOC1 passages).

which the phorbol activates PKC (Abdullah *et al.* 2003). A much stronger degree of stimulation was observed for cells exposed to higher PMA concentrations, as previously shown (Abdullah *et al.* 1997). Mucin secretion elicited by PMA from BAPTA-buffered cells was inhibited significantly: at the lower PMA concentrations BAPTA abolished secretion and at higher concentrations it was diminished relative to EGTA-buffered cells.

To test whether IP_3-mediated signalling might be responsible for the apparent Ca^{2+} dependency of PMA-stimulated secretion, we challenged SLO-permeabilized, EGTA-buffered SPOC1 cells with PMA in the presence of heparin. Figure 6*B* shows that heparin had no effect on the PMA response at any concentration (cf. Fig. 1), suggesting that the resulting secretion of mucin is stimulated by an IP_3-independent mechanism.

Discussion

EGTA has been used for nearly a quarter of a century to control Ca^{2+} in permeabilized secretory cells (Baker & Knight, 1981; Bennett *et al.* 1981). Remarkably, it continues to be the chelator of choice nearly 20 years after the synthesis of BAPTA (Tsien, 1980), a chelator that binds Ca^{2+} nearly 2 orders of magnitude faster despite having a similar affinity (Smith *et al.* 1984; Harrison & Bers, 1987). Theoretically, these kinetics allow BAPTA, but not EGTA, to buffer Ca^{2+} effectively at the mouths of open Ca^{2+} channels, up to limits imposed by the diffusivity of the chelator (Neher, 1986; Stern, 1992; Bauer, 2001; Smith *et al.* 2001). For example, our calculations show that under the conditions of our experiments BAPTA buffers Ca^{2+} ~7-fold closer to the channel than does EGTA (Fig. 2*B*). The faster Ca^{2+} binding kinetics of BAPTA have been exploited successfully to probe the generation of Ca^{2+} transients and/or local gradients that occur in exocytosis, using permeable acetoxymethylester analogues to load, or a micropipette or patch pipette to inject/dialyse, the buffer into the target cells. For example, BAPTA markedly attenuated transmitter release at the squid giant synapse (Adler *et al.* 1991), diminished the amplitude of Ca^{2+} transients in mouse spinal cord neurones (Tymianski *et al.* 1994), and inhibited glucose-induced insulin secretion in pancreatic β-cells (Pertusa *et al.* 1999), while

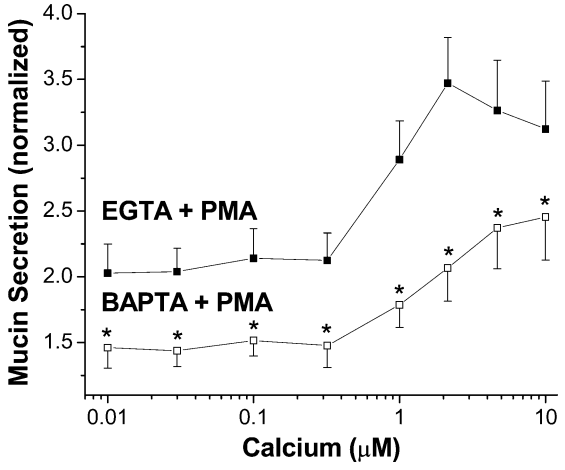

Figure 5. Effects of rapid Ca^{2+} buffering on PMA-induced mucin secretion from SPOC1 cells
SLO-permeabilized cells were exposed to Ca^{2+} (0.01–10 μM) buffered by EGTA or BAPTA, ± 300 nM PMA. Mucin secretion was normalized to that released by 0.1 μM Ca^{2+} in the absence of PMA (for clarity, controls not shown; compare with Fig. 2*C*). Two-way ANOVA indicates a significant reduction by BAPTA, as well as a significant effect of Ca^{2+} with both buffers ($n = 6$ SPOC1 passages).

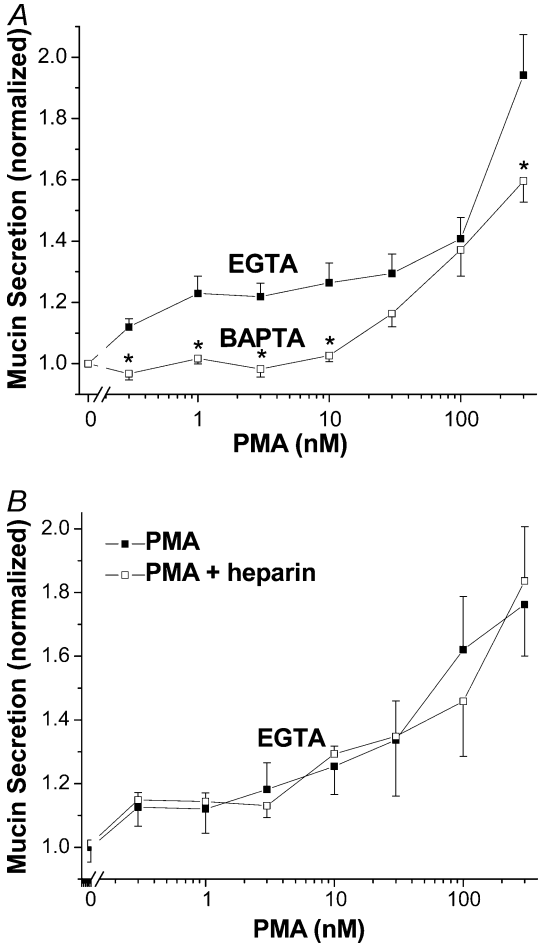

Figure 6. Concentration effects of PMA in SLO-permeabilized SPOC1 cells
A, cells were stimulated with 0.3–300 nM PMA at 0.1 μM Ca^{2+} in EGTA- or BAPTA-buffered solutions. Two-way ANOVA indicates a significant difference between EGTA and BAPTA ($n = 6$ SPOC1 passages). *B*, EGTA-buffered cells were stimulated with 0.3–300 nM PMA at 0.1 μM Ca^{2+} ± heparin (200 μg ml^{-1}). No significant differences were detected ($n = 4$ SPOC1 passages).

EGTA either had smaller or no significant effect. We took advantage of BAPTA's ability to buffer Ca^{2+} in near membrane environments to reveal Ca^{2+} dependency in pathways controlling exocytosis in SPOC1 cells previously thought to be independent of Ca^{2+} (Scott *et al.* 1998).

Ca^{2+} plays a central role in triggering exocytosis in numerous secretory cells; however, the process is particularly poorly defined in airway goblet cells. A useful model is the pancreatic acinar cell where presentation of agonist to the basolateral membrane induces an IP_3-dependent Ca^{2+} release selectively, within the secretory granule region in the apical pole to trigger zymogen granule exocytosis (Ito *et al.* 1997). Agonist-induced Ca^{2+} release in acinar cells is sensitive to heparin, indicating an involvement of IP_3 (Thorn *et al.* 1993*b*), and dialysis of the cell with IP_3 induces transient or oscillatory Ca^{2+} release from IP_3-sensitive intracellular Ca^{2+} stores (Thorn *et al.* 1993*a*; reviewed by Berridge, 1993; Petersen, 1992). In goblet cells, IP_3 releases Ca^{2+} from secretory granules (Nguyen *et al.* 1998).

IP_3-generated Ca^{2+} gradients in SPOC1 cell

IP_3 induced mucin secretion from permeabilized, EGTA-buffered SPOC1 cells in a heparin-sensitive manner (Fig. 1). The magnitude of IP_3-induced mucin release was similar to that induced by 10 μM bulk Ca^{2+} levels. Hence, our results suggest that EGTA allows a local elevation in Ca^{2+} to micromolar levels following its IP_3-mediated release from SPOC1 cell internal stores. This elevation is sufficiently robust and long-lived to initiate and trigger the exocytosis of mucin secretory granules. Mucin release elicited by IP_3 in the presence of BAPTA was inhibited by 69–100% (Figs 3 and 4), compared to EGTA, suggesting that IP_3 releases Ca^{2+} locally, generating Ca^{2+} gradients of a magnitude sufficient to trigger exocytosis. The differences in the mucin secretory response to IP_3 by cells buffered with EGTA or BAPTA appear to be specific to their binding kinetics, and not to be due to non-specific effects of the buffers (Fig. 4).

Ca^{2+} dependency of PMA-induced mucin secretion in SPOC1 cells

PMA and PDBu are classical phorbol ester secretagogues used to activate conventional and novel isoforms of PKC. Although they are often used at, or near, micromolar concentrations, maximal activation of PKC typically occurs at 10–30 nM (Liles *et al.* 1987; Kazanietz *et al.* 1993). Mucin secretion from SPOC1 cells, however, is stimulated maximally by PMA at 300 nM (Abdullah *et al.* 1997); hence, this was the concentration used originally to test the Ca^{2+} independence of PMA effects on mucin release from permeabilized, EGTA-buffered cells (Scott *et al.* 1998; see Introduction). In the study of Fig. 5, the original observation that 300 nM PMA stimulates mucin release from SPOC1 cells over a range of 0.01–30 μM Ca^{2+} was duplicated successfully. When BAPTA was used as the Ca^{2+} buffer in paired experiments, however, mucin secretion was inhibited substantially indicating that, in fact, PMA-induced mucin secretion has a strong Ca^{2+} dependency. Hence, this result supports the notion that regulated exocytosis from SPOC1 cells is Ca^{2+} dependent, as suggested by the Ca^{2+} dependency of regulated exocytotic pathways, in general (see Chapman, 2002; Sudhof, 2002).

In recent years, proteins other than PKC have been shown to possess the C1 domain necessary to be an effective phorbol ester receptor (e.g. see Kazanietz, 2002). Considering that PKC translocation to the membrane saturates between 10 and 30 nM PMA in SPOC1 cells (Abdullah *et al.* 2003), the effects of higher phorbol ester levels in SPOC1 cells are clearly PKC independent and may be due to the obligate, C1 domain, exocytotic accessory protein, ubMUNC13-2 (Koch *et al.* 2000; Rhee *et al.* 2002) that is expressed in these cells. Notably, the PMA concentration–effect studies conducted in BAPTA-based buffers were consistent with this scenario (Fig. 6*A*): at concentrations below 30 nM the effects of PMA in SPOC1 cells buffered by BAPTA were blocked completely, whereas EGTA proved permissive. PMA concentrations > 30 nM effectively stimulated mucin secretion from BAPTA-buffered cells to levels similar to those elicited in EGTA, except for the highest PMA concentration (300 nM) where mucin release in BAPTA was inhibited slightly. We speculate that the stimulatory effects of low PMA levels (≤ 30 nM) in EGTA-buffered cells correspond to the activation of PKC, which most likely phosphorylates MARCKS to initiate the disruption of actin cortical microfilaments (Trifaro *et al.* 2000). The stimulation that occurs in both EGTA- and BAPTA-buffered cells at higher PMA concentrations most likely reflects diffusion distances that are very short, a scenario consistent with the activation of ubMUNC13-2 to prime mucin secretory granules docked at the plasma membrane (Brose *et al.* 2000; Martin, 2002). BAPTA's superior ability to buffer Ca^{2+} near channel openings might explain the generally diminished secretion in BAPTA-buffered cells, relative to EGTA-buffered cells, at high PMA concentrations (Fig. 6*A*). As illustrated in Fig. 2*B*, under the conditions of our experiments BAPTA is expected to buffer Ca^{2+} to below 1 μM beyond a $\sim$50 nm radius of channel openings. Within this radius, Ca^{2+} gradients exceeding 10 μM are expected to develop and are likely to activate Ca^{2+}-dependent proteins. Small distances of this order are appropriate to the plasma and secretory granule membranes as they approach one another during the exocytotic process. Interestingly, these molecular distances may explain why secretion occurred

in the face of rapid Ca^{2+} buffering by BAPTA during stimulation by both IP_3 (Fig. 3) and PMA (Figs 5 and 6).

Chief among the unanswered questions in this scenario are the signal initiating Ca^{2+} release in PMA-treated SPOC1 cells and the source of the Ca^{2+}. PMA activation of PKC in some cells results in a release of Ca^{2+} from internal stores through an undefined mechanism (e.g. see Xuan *et al.* 1994), and such could be the case for SPOC1 cells exposed to PMA concentrations ≤ 30 nM. The extra stimulatory effects of PMA at concentrations > 30 nM, however, indicates a PKC-independent mechanism (Abdullah *et al.* 2003), and the lack of inhibition of PMA-induced secretion by heparin (Fig. 6*B*) effectively excludes IP_3 as the potential signalling molecule. The Ca^{2+} store could be cisternae of the endoplasmic reticulum located near mucin granules, as it is in gonadotrophs (Tse *et al.* 1997), or potentially more interestingly, it could be the mucin secretory granule itself (Nguyen *et al.* 1998; and see Petersen, 1996). A granule-based store raises the intriguing possibility that Ca^{2+} is released from the granule as it interacts physically with the plasma membrane during the pre-exocytotic events involved in granule docking, priming and formation of the exocytotic pore complex. Precedence for such a mechanism exists in yeast where Ca^{2+} is released from the lumen of the vacuole as it interacts with its exocytotic docking site to stimulate the final steps of vacuolar secretion (Peters & Mayer, 1998).

In conclusion, by virtue of its slow binding kinetics EGTA is permissive with respect to the generation of local Ca^{2+} gradients in permeabilized SPOC1 cells, whereas BAPTA is a much more efficient Ca^{2+} buffer and inhibits mucin secretion. Hence, the SPOC1 cell mucin secretory response to PMA we observed previously in permeabilized, EGTA-buffered cells and proposed to be independent of Ca^{2+} does, in fact, possesses a definite Ca^{2+} dependency. What remains to be tested rigorously for Ca^{2+} dependency in other secretory cells are the cAMP-induced responses proposed to be Ca^{2+} independent (Koh *et al.* 2000), as well as other studies in which measurements of whole cell Ca^{2+} and exocytosis failed to exhibit the correspondence expected for Ca^{2+}-dependent events (e.g. Neher & Almers, 1986). A major problem that needs to be resolved with such studies is the measurement of *local* Ca^{2+} – because the studies above were based on the use of fluorescent Ca^{2+} indicators using wide-field epifluorescence microscopy, the small signal from local Ca^{2+} gradients that might have occurred could have been buried in the noise of the larger signal being recorded from the whole cell. Ca^{2+} sparks, for instance, were not observed until the advent of confocal microscopy (Cannell *et al.* 1994). Clearly, before such data can be accepted as indicating genuine Ca^{2+}-independent exocytosis, the measurements need to be repeated using confocal microscopy and the appropriate controls.

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