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Barium and Calcium Stimulate Secretion from Digitonin-Permeabilized Bovine Adrenal Chromaffin Cells by Similar Pathways

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Abstract: We compared the characteristics of secretion stimulated by EGTA-buffered Ba^{2+}- and Ca^{2+}-containing solutions in digitonin-permeabilized bovine adrenal chromaffin cells. Half-maximal secretion occurred at approximately 100 μM Ba^{2+} or 1 μM Ca^{2+}. Ba^{2+}-stimulated release was not due to release of sequestered intracellular Ca^{2+} because at a constant free Ba^{2+} concentration, increasing unbound EGTA did not diminish the extent of release due to Ba^{2+}. The maximal extents of Ba^{2+}- and Ca^{2+}-dependent secretion in the absence of MgATP were identical. MgATP enhanced Ba^{2+}-induced secretion to a lesser extent than Ca^{2+}-induced secretion. Half-maximal concentrations of Ba^{2+} and Ca^{2+}, when added together to cells, yielded approximately additive amounts of secretion. Maximal concentrations of Ba^{2+} and Ca^{2+} when added together to cells for 2 or 15 min were not additive. Tetanus toxin inhibited Ba^{2+}- and Ca^{2+}-dependent secretion to a similar extent. Ba^{2+}, unlike Ca^{2+}, did not activate polyphosphoinositide-specific phospholipase C. These data indicate that (1) Ba^{2+} directly stimulates exocytosis, (2) Ba^{2+}-induced secretion is stimulated to a lesser extent than Ca^{2+}-dependent secretion by MgATP, (3) Ba^{2+} and Ca^{2+} use similar pathways to trigger exocytosis, and (4) exocytosis from permeabilized cells does not require activation of polyphosphoinositide-specific phospholipase C. **Key Words:** ATP—Barium—Calcium—Chromaffin cell—Exocytosis—Tetanus toxin. **TerBush D. R. and Holz R. W.** Barium and calcium stimulate secretion from digitonin-permeabilized bovine adrenal chromaffin cells by similar pathways. *J. Neurochem.* **58,** 680–687 (1992).

Nicotinic agonist- or elevated K^+-induced secretion from bovine adrenal chromaffin cells is critically dependent on influx of extracellular Ca^{2+} and a subsequent rise in intracellular Ca^{2+} (Wilson and Kirshner, 1977; Holz et al., 1982; Kilpatrick et al., 1982; Knight and Kesteven, 1983; Kao and Schneider, 1986). Although many processes are activated by Ca^{2+} within cells [e.g., protein phosphorylation (Amy and Kirshner, 1981; Cote et al., 1986; Lee and Holz, 1986; Pocotte et al., 1986), phospholipase C (Eberhard and Holz, 1987; Sasakawa et al., 1989), and arachidonate release (Frye and Holz, 1984, 1985; Morgan and Burgoyne, 1990)], the process that is responsible for triggering exocytosis is unknown. Extracellular Ba^{2+}, substituted for Ca^{2+}, can enter chromaffin cells through voltage-dependent Ca^{2+} channels (Artalejo et al., 1987; Heldman et al., 1989) and stimulate release of catecholamines (Douglas and Rubin, 1964*a*,*b*). Ba^{2+} also stimulates secretion from other cells (Hales and Milner, 1968; Davidson et al., 1987; van der Merwe et al., 1990). In experiments with intact cells, it is unclear whether intracellular Ba^{2+} directly triggers secretion or whether Ba^{2+} acts indirectly by releasing Ca^{2+} from intracellular stores. This distinction is important because it helps characterize the divalent cation dependency of the secretory pathway. Furthermore, if Ca^{2+} and Ba^{2+} stimulate secretion by identical mechanisms, then only those biochemical processes that are activated by both would be necessary for secretion.

In this study we have expanded on recent observations that Ba^{2+} stimulates secretion from permeabilized chromaffin cells (Izumi et al., 1986; Knight et al., 1988; Heldman et al., 1989). We examined the characteristics of Ba^{2+}- and Ca^{2+}-dependent secretion and phosphoinositide-specific phospholipase C activity in digitonin-permeabilized cells using EGTA buffers to control the

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Abbreviations used: BSA, bovine serum albumin; CBP, calmodulin-binding peptide; GTPγS, guanosine-5′-*O*-thiotriphosphate; KGEP, solution containing 140 m*M* potassium glutamate, 10 m*M* EGTA, 20 m*M* PIPES (pH 6.6), and 5 mg/ml BSA; PSS, physiological salt solution.

amounts of free Ba^{2+} and free Ca^{2+} available to stimulate exocytosis. The interactions between Ba^{2+} and Ca^{2+}, the effects of tetanus toxin, and the effects of MgATP indicate that Ba^{2+} directly stimulates secretion by a pathway similar to that stimulated by Ca^{2+}. Because Ba^{2+} is a poor activator of polyphosphoinositide-specific phospholipase C and calmodulin-dependent processes, these experiments limit the biochemical reactions necessary for exocytosis.

EXPERIMENTAL PROCEDURES

Materials

All chemicals, except as noted, were purchased from Sigma (St. Louis, MO, U.S.A.). Calf skin collagen and tetanus toxin were acquired from Calbiochem (San Diego, CA, U.S.A.). Amphotericin B (Fungizone) was obtained from Squibb (Princeton, NJ, U.S.A.). [^{3}H]Norepinephrine was acquired from Amersham (Arlington Heights, IL, U.S.A.) and *myo*-[2-^{3}H]inositol was from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). Culture plates were purchased from Costar (Cambridge, MA, U.S.A.) and scintillation fluid was from ICN Biomedicals (Irvine, CA, U.S.A.). Digitonin was from Fluka (Ronkonkoma, NY, U.S.A.). Culture medium was from Whitaker Bioproducts (Walkersville, MD, U.S.A.) and Dowex AG1-X8 resin was from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

General tissue culture and experimental conditions

Primary dissociated cells from bovine adrenal medulla were prepared and maintained as monolayer cultures as previously described by Holz et al. (1982). Chromaffin cells used for determining release of radiolabeled inositol phosphates (Fig. 6) were purified by differential plating (Waymire et al., 1983). Cells were cultured as monolayers in 6.4-mm diameter culture wells at 500,000 cells/cm^2. Culture wells were coated with 5 μg of calf skin collagen/cm^2 prior to cell plating. Three to four days after plating, culture medium was replaced with fresh medium not containing Fungizone. Immediately before an experiment, cells were washed three times for 15 min with physiological salt solution (PSS) [145 m*M* NaCl, 5.6 m*M* KCl, 2.2 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 5.6 m*M* glucose, 15 m*M* HEPES (pH 7.4), 0.57 m*M* sodium ascorbate, 5 mg/ml bovine serum albumin (BSA)]. All experiments were performed at 25°C on cells cultured 4–8 days.

Assay of secretion

Before an experiment, cells were incubated for 4–24 h in culture medium containing [^{3}H]norepinephrine (2 μCi/ml) supplemented with 0.1 mg/ml of ascorbate. After washing with PSS, an experiment was initiated by rendering cells leaky with a KGEP solution [140 m*M* potassium glutamate, 10 m*M* EGTA, 20 m*M* piperazine-*N,N'*-bis(2-ethanesulfonic acid (PIPES) (pH 6.6), 5 mg/ml BSA] containing 20 μ*M* digitonin, 2 m*M* ATP, and 1 m*M* free Mg^{2+} for 4–6 min. In experiments where the effects of ATP on secretion were examined (Figs. 2, 3, and 4A), the cells were rendered leaky in the absence of ATP and Mg^{2+}. The permeabilization solution was removed and replaced with a KGEP solution either containing or lacking 2 m*M* ATP (1 m*M* free Mg^{2+}) and various amounts of $CaCl_2$ and/or $BaCl_2$ buffered to yield free concentrations of 0–10 μ*M* for Ca^{2+} and 0–3 m*M* for Ba^{2+} (see below). After 2–30 min the supernatants were removed and the cells disrupted with 1% Triton X-100. The amount of radioactivity in the supernatant and the lysed cells was determined by liquid scintillation spectrometry with Universol ES as scintillant. Secretion was the amount of [^{3}H]norepinephrine released (*R*) divided by the sum of that released plus that remaining in the cells (*C*) for a given data point (i.e., percent secretion = $[R/(R + C)] \times 100$).

The free concentrations of Mg^{2+}, Ca^{2+}, and Ba^{2+} were calculated according to the computer program developed by Chang et al. (1988) using the affinity (stability) constants and protonation constants from Bjerrum et al. (1957). The affinity constants used were:

Metal	$\text{Log}K^{M}_{MHL}$	$\text{Log}K_1$
Ca^{2+}	5.33	11.00
Ba^{2+}	4.26	8.41
Mg^{2+}	3.37	5.21

The protonation constants used were: H_4L, 2; H_3L^-, 2.68; H_2L^{2-}, 8.85; HL^{3-}, 9.46 (expressed as log base 10). A sample KGEP solution (10 m*M* EGTA, 2 m*M* ATP) containing 1 m*M* free Mg^{2+}, 10 μ*M* free Ca^{2+}, and 1 m*M* free Ba^{2+} contained total concentrations of 2.55 m*M* $MgCl_2$, 7.16 m*M* $CaCl_2$, and 3.17 m*M* $BaCl_2$.

In most secretion experiments (Figs. 2–5; Table 2) D600 (100 μ*M*), a Ca^{2+} channel blocker, was included to ensure that secretion stimulated by Ba^{2+} came from permeabilized chromaffin cells and not from a small population of unpermeabilized cells. D600, 100 μ*M*, reduced Ba^{2+}-induced secretion from intact cells by 80–90% which indicates that D600 is an effective blocker of Ba^{2+} influx in nonpermeabilized cells. D600, 100 μ*M*, reduced Ba^{2+}-induced secretion from digitonin-permeabilized cells by only 18% ± 2% (n = 5 experiments) and had no effect on Ca^{2+}-dependent secretion from permeabilized cells. Several experiments without D600 (Fig. 1 and Table 1) are included in the article to demonstrate that D600 does not qualitatively change the results. Previous studies may have overestimated the amount of Ba^{2+}-dependent secretion from permeabilized cells by not blocking Ba^{2+}-stimulated release from remaining intact cells (Izumi et al., 1986; Knight et al., 1988).

Assay of ^{3}H-inositol phosphates

Cellular inositol-containing lipids were labeled by incubation for 48 h with *myo*-[2-^{3}H]inositol (20 μCi/ml) in Eagle's minimum essential medium (which contained 11 μ*M* unlabeled *myo*-inositol) supplemented with 10% (vol/vol) dialyzed fetal bovine serum, L-glutamine (2 m*M*), penicillin (100 U/ml), and streptomycin (100 μg/ml). Before an experiment, labeling medium was removed and the cells were washed twice for 10 min with PSS. Cells were then handled similarly to a secretion experiment except the incubation solution was assayed for released radiolabeled inositol phosphates. Tritiated inositol phosphates were determined by diluting the incubation solutions with 3 ml of H_2O and applying them to freshly poured columns (0.3 ml) of Dowex AG1-X8 resin (formate form) similar to the method of Eberhard et al. (1990). Free inositol was eluted with 8 × 1 ml washes with 60 m*M* sodium formate/5 m*M* borax. These washes were usually discarded. Inositol monophosphate was eluted with 3 × 1 ml washes with 0.2 *M* ammonium formate/0.1 *M* formic acid. The 3-ml fraction was collected in a 20-ml scintillation vial and was counted with 15 ml of Universol ES. The columns were then washed 4 × 1 ml with 0.2 *M* ammonium formate/0.1 *M* formic acid and the solution discarded. Inositol bisphosphate was eluted with 4 × 1 ml and 4 × 0.75 ml washes

with 0.4 *M* ammonium formate/0.1 *M* formic acid and collected into two separate 20-ml scintillation vials as 3.5-ml fractions to which was added 17 ml of scintillation fluid. The columns were then washed 4 × 1 ml with 0.4 *M* ammonium formate/0.1 *M* formic acid and the solution discarded. Inositol trisphosphate was eluted with 4 × 1 ml washes with 1.0 *M* ammonium formate/0.1 *M* formic acid. The 4-ml fraction was collected in a 20-ml scintillation vial and was counted with 15 ml of scintillation fluid. The amount of radioactivity in the individual fractions was then determined by scintillation spectrometry. The data for Fig. 6 show the sum of the individually counted forms of released labeled inositol phosphates.

Representative data from individual experiments are plotted as means ± SEM and error bars smaller than the plot symbols were omitted. Experiments were repeated at least twice and usually three or more times. Significance between groups was determined by Student's *t* test ($p < 0.05$). When multiple comparisons were made, analysis of variance was used to determine if the means were different ($p < 0.05$) and pairwise comparisons were performed using Tukey's Method ($p < 0.05$).

RESULTS

Ba^{2+}-stimulated secretion from digitonin-permeabilized chromaffin cells

Ba^{2+} stimulated half-maximal secretion at 100 μM and maximal secretion at 1 m*M* in digitonin-permeabilized cells (Fig. 1). Ca^{2+} and Ba^{2+} stimulated secretion at significantly different concentrations with half-maximal secretion occurring at a 100-fold greater Ba^{2+} concentration.

Because micromolar Ca^{2+} stimulates secretion from permeabilized cells it was necessary to determine whether Ba^{2+} directly stimulated secretion or stimulated secretion indirectly by releasing intracellular Ca^{2+}. At 0.6 m*M* free Ba^{2+} (10 m*M* total EGTA, 1 m*M* free Mg^{2+}, 2 m*M* ATP) there is 4.42 m*M* free EGTA. To ensure that Ba^{2+} was acting directly and not by release

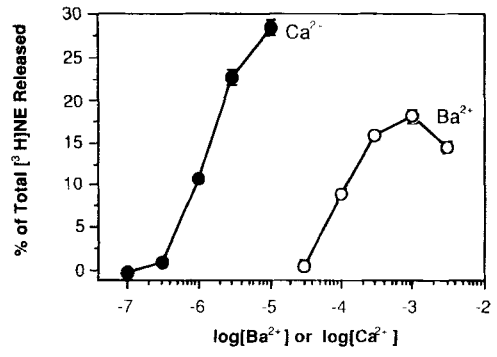

FIG. 1. Ba^{2+} and Ca^{2+} dose–response curves of secretion from digitonin-permeabilized chromaffin cells. Cells were permeabilized for 4 min with 20 μM digitonin in KGEP in the presence of 2 m*M* ATP and 1 m*M* free Mg^{2+}. The permeabilization solution was removed and KGEP media containing various free concentrations of Ba^{2+} or Ca^{2+} was added for 15 min in the continuing presence of the same concentrations of ATP and Mg^{2+}. The percentage of total labeled catecholamine released into the incubation medium was determined. There were three wells per group.

TABLE 1. *Effect of EGTA buffer strength on Ba^{2+}-stimulated secretion from digitonin-permeabilized chromaffin cells*

[Ba^{2+}] m*M* (free)	[EGTA] m*M* (total)	[EGTA] m*M* (free)	Percent Ba^{2+}-dependent secretion
0.6	1	0.44	12.7 ± 0.4
0.6	3	1.33	12.9 ± 0.5
0.6	10	4.42	11.6 ± 0.3

Cells were permeabilized for 4 min with 20 μM digitonin in KGEP in the presence of 2 m*M* ATP and 1 m*M* free Mg^{2+}. The permeabilization solution was removed and KGEP (1, 3, or 10 m*M* EGTA) containing 0 or 0.6 m*M* free Ba^{2+} in the presence of 2 m*M* ATP and 1 m*M* free Mg^{2+} was added for 15 min. The percentage of total labeled catecholamine released into the incubation medium was determined. Secretion in the absence of Ba^{2+} was between 2.2 and 2.4%. There were four wells per group.

of intracellular Ca^{2+}, secretion was stimulated by a constant amount of free Ba^{2+} while the total concentration of EGTA was increased from 1 to 10 m*M* (Table 1). Because Ca^{2+} has a much higher affinity for EGTA than does Ba^{2+}, any released Ca^{2+} should be chelated by the increasing concentration of free EGTA. Ba^{2+}-dependent secretion was unaffected as the free EGTA concentration increased from 0.44 to 4.42 m*M*. Thus, Ba^{2+} acts directly to release catecholamines.

Effect of ATP on secretion stimulated by Ca^{2+} and Ba^{2+} from permeabilized cells

Shortly after permeabilization there is a component of Ca^{2+}-dependent secretion that occurs in the absence of MgATP in the medium (Holz et al., 1989). This secretion is rapid and labile and may represent an effect of ATP in intact cells that primes the cell to secrete. In addition, there is a slower, more stable component of secretion that requires MgATP in the medium (Knight and Baker, 1982; Brooks and Treml, 1983; Dunn and Holz, 1983; Wilson and Kirshner, 1983; Morita et al., 1988; Holz et al., 1989). In the absence of medium MgATP, maximal concentrations of Ba^{2+} and Ca^{2+} stimulated secretion to the same extent (Fig. 2) and with a similar time course (Fig. 3). Although MgATP increased secretion stimulated by either Ba^{2+} or Ca^{2+}, it enhanced Ba^{2+}-induced secretion to a lesser degree than Ca^{2+}-induced secretion (Figs. 2 and 3). MgATP enhanced Ba^{2+}-stimulated secretion 50–100% and Ca^{2+}-stimulated secretion 200–400%.

Effects on secretion of the combination of Ca^{2+} and Ba^{2+} in digitonin-permeabilized chromaffin cells

The interaction between Ca^{2+}- and Ba^{2+}-stimulated secretion in the presence or absence of MgATP was examined to determine whether secretion stimulated by the cations was additive when both were present. Both in the presence and absence of MgATP, secretion stimulated by medium containing half-maximal concentrations of both Ca^{2+} (1 μM) and Ba^{2+} (100 μM) was approximately additive at 15 min (Fig. 4). Secretion stimulated by medium containing maximal concen-

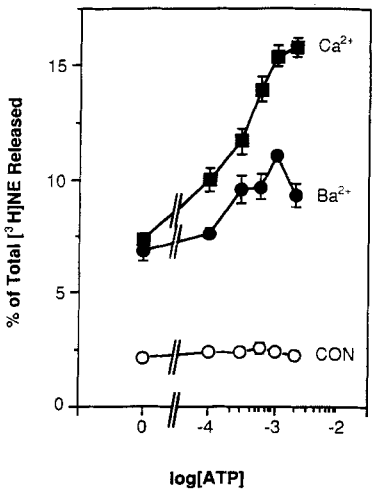

FIG. 2. ATP dose–response curve of secretion stimulated by Ba^{2+} or Ca^{2+} from digitonin-permeabilized cells. Cells were permeabilized for 4 min with 20 μM digitonin in KGEP in the absence of divalent cations and ATP and in the presence of 100 μM D600. The permeabilization solution was removed and KGEP incubation media containing no added Ba^{2+}/Ca^{2+} (CON), 1 mM free Ba^{2+}, or 10 μM free Ca^{2+} were added for 15 min in the presence of increasing concentrations of ATP and 1 mM free Mg^{2+}. The percentage of total labeled catecholamine released into the incubation medium was determined. There were three wells per group.

trations of both Ca^{2+} (10 μM) and Ba^{2+} (1 mM) was no greater than that stimulated by a maximal concentration of Ca^{2+} alone at 15 min (Fig. 4). The effects of 10 μM Ca^{2+} and 1 mM Ba^{2+} were also nonadditive after a 2-min incubation (Table 2).

Effect of tetanus toxin on secretion stimulated by Ca^{2+} and Ba^{2+} from digitonin-permeabilized chromaffin cells

Tetanus toxin is a potent inhibitor of secretion in neurons and chromaffin cells and is thought to interact with critical sites in the Ca^{2+}-dependent exocytotic pathway. Tetanus toxin (50 μg/ml) inhibited Ca^{2+}- and Ba^{2+}-stimulated secretion to a similar extent (Fig. 5). Ca^{2+}-dependent secretion was inhibited 72% and Ba^{2+}-dependent secretion was inhibited 67% by tetanus toxin. Thus, tetanus toxin-sensitive components are common to the secretory pathways stimulated by Ca^{2+} and Ba^{2+}.

Effects of Ba^{2+} and Ca^{2+} on biochemical processes

Because Ba^{2+} and Ca^{2+} stimulated secretion similarly in digitonin-permeabilized cells, we examined their effects on biochemical processes that might be required for the secretory response. We reasoned that if the biochemical mechanism of secretion stimulated by Ba^{2+} and Ca^{2+} is similar, then a process activated by only one of the two cations could not be an obligatory component of the mechanism of exocytosis. In the present study we found that Ba^{2+} (1 mM) did not stimulate the release of inositol phosphates in permeabilized cells (Fig. 6). Ba^{2+} almost completely blocked the effect of the guanosine-5′-*O*-thiotriphosphate (GTPγS) to cause release of inositol phosphates (92% inhibition, $p < 0.01$). In contrast, Ca^{2+} and GTPγS when they were present together stimulated a somewhat greater than additive (not statistically significant) release of inositol phosphates (Fig. 6). Ba^{2+} had no effect on the Ca^{2+}-dependent release of inositol phosphates.

We also examined whether inhibiting calmodulin-dependent processes with a calmodulin-binding peptide (CBP) derived from the multifunctional Ca^{2+}/calmodulin-dependent protein kinase would decrease secretion stimulated by Ba^{2+}. When secretion was stimulated from permeabilized cells with 1 mM free Ba^{2+} in the presence of 10 μM CBP there was no inhibition of secretion (29.8% ± 0.9% in the absence of CBP versus 27.4% ± 0.8% in the presence of CBP, n = 3). The CBP also had no inhibitory effect on secretion stimulated by 0.1 mM Ba^{2+} (data not shown). The CBP (3 μM) did not inhibit secretion stimulated by 1 μM Ca^{2+} even though it inhibited Ca^{2+}-dependent protein phosphorylation (TerBush and Holz, 1990).

DISCUSSION

Ba^{2+} and Ca^{2+} stimulate secretion by similar mechanisms

This study demonstrates that Ba^{2+} can directly stimulate secretion. In permeabilized chromaffin cells, Ba^{2+} does not act by releasing Ca^{2+} from intracellular stores (Table 1). The data strongly suggest that Ba^{2+} and Ca^{2+} stimulate secretion by similar mechanisms: 1. When digitonin-permeabilized cells were incubated with maximal concentrations of both Ba^{2+} and Ca^{2+}, the maximal amount of secretion was no greater than with Ca^{2+} alone at 15 min (Fig. 4) and only slightly greater

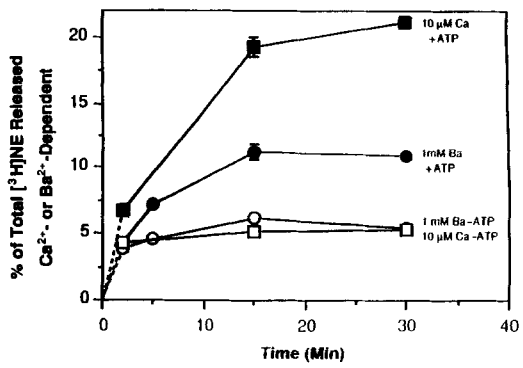

FIG. 3. Time course of secretion stimulated in the presence or absence of Ba^{2+} or Ca^{2+} in the presence or absence of ATP from digitonin-permeabilized cells. Cells were permeabilized for 4 min with 20 μM digitonin in KGEP in the absence of divalent cations and ATP. The permeabilization solution was removed and KGEP incubation media containing no added Ba^{2+}/Ca^{2+}, 1 mM free Ba^{2+}, or 10 μM free Ca^{2+} were added for various times in the presence of 1 mM free Mg^{2+} and 100 μM D600 and the presence or absence of 2 mM ATP. The percentage of total labeled catecholamine released into the incubation medium was determined. There were four wells per group.

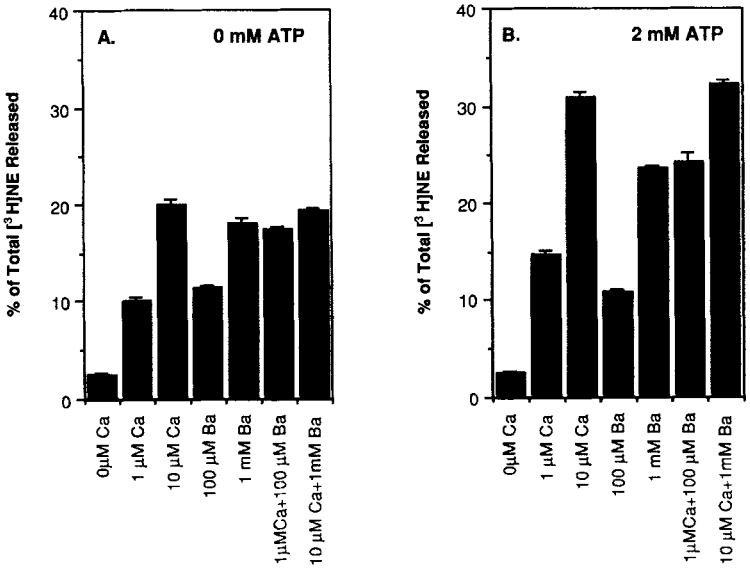

FIG. 4. Interaction between Ba^{2+}- and Ca^{2+}-stimulated secretion from digitonin-permeabilized cells. Cells were permeabilized for 4 min with 20 μM digitonin in KGEP in the presence of 1 m*M* free Mg^{2+} and in the absence (**A**) or presence (**B**) of 2 m*M* ATP. The permeabilization solution was removed and KGEP incubation media containing 100 μM D600, 1 m*M* free Mg^{2+}, and various free concentrations of Ca^{2+} or Ba^{2+} was added for 15 min in the absence (A) or presence (B) of 2 m*M* ATP. The percentage of total labeled catecholamine released into the incubation medium was determined. Secretion in the presence of 10 μM Ca^{2+} and 1 m*M* Ba^{2+} is not significantly different from secretion in the presence of 10 μM Ca^{2+} alone in panels A and B. There were three wells per group.

than with Ca^{2+} alone at 2 min (Table 2). Thus, Ba^{2+} and Ca^{2+} probably compete to activate a common step(s) in the pathway for exocytosis. Ca^{2+} and Ba^{2+} are not simply competing for a limiting pool of chromaffin granules available for secretion because secretion is nonadditive when the available pool is not limiting (Table 2). 2. Tetanus toxin similarly inhibited secretion induced by Ba^{2+} or Ca^{2+} (Fig. 5). Thus, tetanus toxin interacts with similar or identical components of the secretory pathway stimulated by either Ba^{2+} or Ca^{2+}. Botulinum toxin type D also inhibits both Ca^{2+}-dependent secretion stimulated by acetylcholine or depolarization and Ba^{2+}-dependent secretion in intact bovine chromaffin cells (Knight et al., 1985). 3. Both Ba^{2+} and Ca^{2+} display secretion that is independent and dependent of ATP in the medium (Figs. 2 and 3).

We had previously demonstrated in permeabilized chromaffin cells that Ca^{2+} stimulates both ATP-independent and ATP-dependent secretion (Holz et al., 1989). Although Ca^{2+} and Ba^{2+} both caused equal

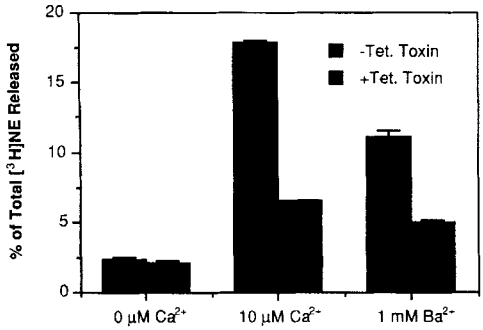

FIG. 5. Effect of tetanus toxin on Ba^{2+}- and Ca^{2+}-stimulated secretion from permeabilized chromaffin cells. Cells were incubated for 6 min with KGEP containing 2 m*M* ATP, 1 m*M* free Mg^{2+}, 100 μM D600, 2 m*M* dithiothreitol (DTT), and 20 μM digitonin in the presence or absence 50 μg/ml of tetanus toxin. The tetanus toxin was activated by incubation in 200 m*M* DTT for 10 min at 30°C before dilution into the KGEP permeabilization solution. The permeabilization solution was removed and KGEP containing the same concentrations of ATP, Mg^{2+}, and D600 and either no additional divalent cations, 10 μM free Ca^{2+}, or 1 m*M* free Ba^{2+} was added for 10 min. The percentage of the total labeled catecholamine released into the incubation medium was determined. There were four wells per group.

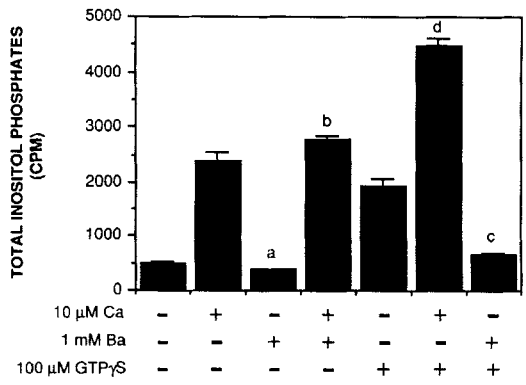

FIG. 6. Effects of Ca^{2+}, Ba^{2+}, and GTPγS on release of inositol phosphates. Cells, prelabeled for 48 h with 20 μCi/ml *myo*-[2-^{3}H]inositol, were permeabilized for 4 min with KGEP containing 20 μM digitonin in the presence or absence of 100 μM GTPγS. The permeabilization solution was removed and KGEP containing 2 m*M* ATP and 1 m*M* free Mg^{2+} and either 10 μM free Ca^{2+}, 1 m*M* free Ba^{2+}, or no further additions for 15 min. The supernatant was removed and the amount of ^{3}H-inositol phosphates released was determined. D600 was omitted from the solutions to avoid any possible effect on phosphatidylinositol-specific phospholipase C activity. Means are different by analysis of variance, $p < 0.01$. There were three wells per group. Significance between paired groups was determined by Tukey's method. [a]Not significantly different compared to 0 μM Ca^{2+} alone; [b]not significantly different compared to 10 μM Ca^{2+} alone; [c]significantly less release compared to GTPγS alone $p < 0.01$; [d]not significantly greater than the sum of release by 10 μM Ca^{2+} and GTPγS alone.

TABLE 2. *Interaction between Ba^{2+}- and Ca^{2+}-stimulated secretion from digitonin-permeabilized cells after 2 min of stimulation*

Incubation solution	Experiment 1	Experiment 2	Experiment 3
0 μM Ca^{2+}	1.5 ± 0.1	2.5 ± 0.1	2.2 ± 0.1
10 μM Ca^{2+}	12.1 ± 0.2	13.6 ± 0.9	16.8 ± 0.7
1 mM Ba^{2+}	9.9 ± 0.5	11.4 ± 0.6	12.3 ± 0.2
10 μM Ca^{2+} + 1 mM Ba^{2+}	13.6 ± 0.8 (20.5)	15.2 ± 0.6 (22.5)	19.1 ± 0.4 (26.9)

Cells were permeabilized for 4 min with 20 μM digitonin in KGEP in the presence of 2 mM ATP, 1 mM free Mg^{2+}, and 100 μM D600. The permeabilization solution was removed and KGEP incubation media containing 100 μM D600, 1 mM free Mg^{2+}, and various free concentrations of Ca^{2+} or Ba^{2+} was added for 2 min in the presence of 2 mM ATP and 1 mM free Mg^{2+}. The percent of total labeled catecholamine released into the incubation medium was determined. The numbers in parentheses indicate the amount of secretion which would be expected if the amount of release stimulated by Ca^{2+} and Ba^{2+} were strictly additive. There were three wells per group.

amounts of ATP-independent secretion, Ca^{2+} stimulated ATP-dependent secretion more than did Ba^{2+}. These data are consistent with there being more than one Ca^{2+}- or Ba^{2+}-requiring site in the secretory pathway. Indeed, recent experiments suggest that Ca^{2+} enhances the ability of ATP to prime secretion independently of its ability to stimulate the final steps of exocytosis (Bittner and Holz, manuscript in preparation). Ba^{2+} may not substitute effectively for Ca^{2+} in the ATP-requiring step(s).

Additional complexity in the divalent ion effects on secretion was revealed by the interaction between Ca^{2+} and Ba^{2+}. Secretion from digitonin-permeabilized cells stimulated by the combination of Ba^{2+} (100 μM) and Ca^{2+} (1 μM) (half-maximal concentrations) was equal to the sum of the secretory responses to each alone in both the presence and absence of ATP. If there is one divalent ion binding site for triggering exocytosis, then subadditive affects are expected. However, the Ca^{2+} and Ba^{2+} activation curves for secretion have Hill coefficients of >1.7 in digitonin-permeabilized cells, indicating that the ions act at at least two sites (TerBush and Holz, unpublished observations). A Hill coefficient of approximately 2 for Ca^{2+} in the secretory response was first demonstrated in electropermeabilized chromaffin cells (Knight and Baker, 1982). Thus, additive effects of Ca^{2+} and Ba^{2+} at suboptimal concentrations of each may reflect positive cooperativity between the divalent ions.

The extent of secretion stimulated by Ba^{2+} from permeabilized cells was less than that from intact cells. Typically, a 15-min incubation with 1 mM Ba^{2+} induced 30–40% secretion from intact cells (in KGEP without digitonin) and 10–20% secretion after cells are permeabilized for 4 min with digitonin. It is possible that essential cytosolic factors required for Ba^{2+}-stimulated secretion rapidly leave permeabilized cells. A similar decrease in Ca^{2+}-dependent secretion occurs with time after permeabilization and may indicate that cytosolic factors common to Ba^{2+}- and Ca^{2+}-dependent secretion are leaving (Dunn and Holz, 1983; Sarafian et al., 1987; Holz et al., 1989).

Activation of phospholipase C is not necessary for secretion

Ba^{2+}, unlike Ca^{2+}, did not activate phospholipase C in digitonin-permeabilized chromaffin cells. These results are consistent with findings in intact chromaffin cells that Ba^{2+} alone has no effect on the release of inositol phosphates although Ca^{2+} influx stimulated by nicotinic agonist or depolarization induced their release (Eberhard and Holz, 1987). The results of the present study support the conclusion that the activation of phospholipase C is not necessary for exocytosis (Eberhard et al., 1990). The inhibition of GTPγS-stimulated release of inositol phosphates by Ba^{2+} in permeabilized chromaffin cells may explain the inhibition by Ba^{2+} of receptor-mediated phospholipase C activity in intact chromaffin cells (Eberhard and Holz, 1987) which is likely mediated by a GTP-binding protein.

Other issues

The apparent similarity between Ba^{2+}- and Ca^{2+}-dependent secretion also makes it unlikely that a calmodulin-dependent process is required for exocytosis because even millimolar Ba^{2+} does not activate calmodulin (Chao et al., 1984). Indeed, when secretion was stimulated from permeabilized cells with 1 mM free Ba^{2+} in the presence of 10 μM of a calmodulin-binding peptide derived from the multifunctional Ca^{2+}/calmodulin-dependent protein kinase, there was no inhibition of secretion. The calmodulin-binding peptide also did not inhibit Ca^{2+}-dependent secretion from permeabilized cells at concentrations effective at inhibiting Ca^{2+}-stimulated phosphorylation of tyrosine hydroxylase (TerBush and Holz, 1990). Thus, it is unlikely that a calmodulin-dependent process is required for secretion. A similar conclusion was reached concerning secretion from PC12 cells (Matthies et al., 1988).

It has recently been reported that one of the annexins, p36, enhances secretion from digitonin-permeabilized chromaffin cells (Ali et al., 1989). Annexins are Ca^{2+} and lipid binding proteins. We know of no information concerning the interaction of Ba^{2+} with p36.

Although there are weak interactions of Ba^{2+} with other annexins (Creutz et al., 1979; Südhof et al., 1982; Zaks and Creutz, 1990), there are insufficient data to determine whether annexins could be a common site of action of Ca^{2+} and Ba^{2+} in triggering exocytosis.

Comparison to previous work

Ba^{2+} and Ca^{2+} trigger secretion by similar mechanisms in permeabilized sheep anterior pituitary cells (van der Merwe et al., 1990). However, the conclusion that Ba^{2+} and Ca^{2+} stimulate secretion by similar mechanisms in chromaffin cells is in disagreement with the conclusion from a recent publication (Heldman et al., 1989). The additive effects of Ba^{2+}-induced secretion and nicotine-induced secretion in the presence of Ca^{2+} from intact cells provided the strongest support for this notion. However, it is unclear from the data whether the actual intracellular concentrations of Ca^{2+} or Ba^{2+} were saturating the secretory response. The study also included an experiment with digitonin-permeabilized cells showing additive effects of Ba^{2+} and Ca^{2+} in EGTA-containing solution. However, the equilibrium between Ba^{2+} and EGTA was not taken into account in the solutions rendering uncertain the pH and concentrations of free Ba^{2+} and Ca^{2+}.

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