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Comparison of Exocytotic Mechanisms between Acetylcholine- and Catecholamine-Containing Vesicles in Rat Pheochromocytoma Cells

Tei-ichi Nishiki,*,†,[1] Yoko Shoji-Kasai,*,[1] Mariko Sekiguchi,* Satoshi Iwasaki,‡ Konosuke Kumakura,† and Masami Takahashi*,‡,[2]

**Mitsubishi Kasei Institute of Life Sciences, Machida, Tokyo 194, Japan; †Life Science Institute, Sophia University, Chiyoda-ku, Tokyo 102, Japan; and ‡Department of Life Sciences (Biology), Graduate School of Arts and Sciences, The University of Tokyo, Meguro-ku, Tokyo 153, Japan*

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The molecular mechanisms of exocytosis from two types of secretory organelles, synaptic-like microvesicles and secretory vesicles, were compared by measuring acetylcholine (ACh) and catecholamine (CA) release from a newly isolated PC12 subclone, PC12-C3 which contains a high level of Ach. Digitonin-permeabilized PC12-C3 cells released both transmitters with similar Ca^{2+}-dependency. Ca^{2+}-evoked ACh and CA release from permeabilized cells were increased in the presence of MgATP, suggesting the existence of a MgATP-dependent priming step prior to the Ca^{2+}-triggered fusion step in both ACh release and CA release. The non-hydrolyzable analogue of GTP, guanosine 5′-(γ- thio)triphosphate (GTPγS), produced both ACh and CA release from permeabilized cells in the absence of Ca^{2+}. Pretreatment with a phorbol ester which activates protein kinase C, potentiated depolarization-induced ACh and CA release from unpermeabilized cells. These results indicate that exocytosis from two distinct vesicle populations are mediated by the same basic molecular mechanisms.

Neurotransmitter release from presynaptic nerve terminals is the primary output from neurons and elucidation of its molecular mechanism is important to understand the basis of synaptic transmission. Amino acid neurotransmitters such as glutamate, glycine and γ- aminobutyrate, and "classical" neurotransmitters including acetylcholine (ACh) and catecholamines (CAs) are stored in small synaptic vesicles (SSVs) of typically 50 nm in diameter (1). On the other hand, peptidic neurotransmitters are present in larger vesicles with an electron-dense core, the so-called large dense-core vesicles (LDCVs) (1). When an action potential reaches the nerve terminal, the presynaptic plasma membrane depolarizes and voltage-gated Ca^{2+} channels open at the active zone. The ensuing rise in intracellular Ca^{2+} triggers the release mechanism, and SSVs and LDCVs release their contents into the synaptic cleft by exocytosis. A large number of proteins potentially involved in exocytosis have been identified and characterized, however, their precise roles in exocytosis are still incompletely understood (2).

Adrenal chromaffin cells and rat pheochromocytoma PC12 cells synthesize and store CAs in secretory vesicles which have many characteristics in common with neuronal LDCVs, and release the contents by Ca^{2+}-dependent exocytosis (3, 4). Since it is quite easy to obtain a large amount of almost homogeneous cellular preparations, chromaffin cells have been widely used to study the molecular mechanisms of exocytosis with biochemical techniques (4). *In vitro* model systems of exocytosis have been successfully made by exposing these cells to a transient electric field or a mild detergent such as digitonin to permeabilize cellular membranes (5, 6). Exocytosis of CAs occurs in the membrane-permeabilized cells only if extracellular free Ca^{2+} ($> 10^{-6}$ M) is present. It is possible to change the intracellular conditions of the permeabilized cells, by changing the extracellular medium, and introducing various proteins, peptides and antibodies which may modify certain processes. By using these systems, it has been shown that there are at least two sequential steps, a MgATP-dependent priming step and a subse-

[1] The first two authors contributed equally to this work.

[2] To whom correspondence should be addressed. Fax: +81-427-24-6314. E-mail: masami@libra.ls.m-kagaku.co.jp.

Abbreviations used in this paper: ACh, acetylcholine; CA, catecholamine; SSVs, small synaptic vesicles; LDCVs, large dense-core vesicles; SLMVs, synaptic-like microvesicles; GTPγS, guanosine 5′-(γ-thio)triphosphate; PMA, phorbol myristate acetate; ECD, electrochemical detector.

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quent MgATP-independent Ca^{2+}-triggered fusion step before the exocytosis of secretory vesicles (7, 8). Moreover, various novel proteins thought to be involved in these steps have been identified and protein-protein interactions occurring in the MgATP-dependent step have been characterized using membrane-permeabilized cells (9-17).

In spite of these advantages, there is also a constraint to using these cells as a model system for the exocytosis of neuronal SSVs. Secretory vesicles are distinct from SSVs in many aspects (1). Secretory vesicles are larger than SSVs and much more heterogeneous in size. In addition, the biochemical properties of secretory vesicles are different from those of SSVs. For example, synaptophysin and rab3A, which are abundant in SSVs, are not present at significant concentrations in the membrane of secretory vesicles (18, 19). Following exocytosis, SSVs undergo local endocytotic recycling and can be refilled with classical neurotransmitters in the nerve terminal. In contrast, secretory vesicles are not reused at the release site. Thus, it is important to know whether the molecular mechanisms of exocytosis of the contents of SSVs and secretory vesicles are different. To address this question, we have utilized rat pheochromocytoma PC12 cells. In addition to CAs, PC12 cells synthesize and accumulate ACh in another population of small vesicles defined as synaptic-like microvesicles (SLMVs) which seem to be much more closely related morphologically and biochemically to SSVs than secretory vesicles (20-23). In the present study, we compared the properties of exocytosis of SLMVs and secretory vesicles by measuring ACh and CA release from PC12 cells. We found that the molecular mechanisms of exocytosis are essentially the same between these two distinct types of vesicles.

MATERIALS AND METHODS

Cell culture. PC12 cells were maintained as previously described (24). For single cell cloning of PC12-C3, after dissociation of the colonies, cells were picked up individually with micropipettes and seeded separately in multi-well tissue culture plates, and the clone containing the largest amount of ACh was selected. PC12-C3 cells were plated onto polyethylenimine-coated 16-mm 4-well multidishes (Nunc) at 5×10^5 cells/cm^2, and cultured for one day before use.

Secretion of ACh and CA from permeabilized PC12 cells. Cell permeabilization was performed at 37°C by the method described originally by Peppers and Holz (25) with slight modifications (26). The culture medium was removed and the cells were rinsed twice with 1 ml of a Ca^{2+}-free Locke's solution (5 mM Hepes-NaOH buffer, pH 6.8 containing 156 mM NaCl, 5.6 mM KCl, 0.2 mM EGTA, 3.6 mM $NaHCO_3$, and 5.6 mM glucose). The cells were permeabilized by an incubation for 5 min with 250 μl of 8 μM digitonin (Calbiochem-Novabiochem) in KGEP (20 mM Pipes-NaOH buffer, pH 6.8 containing 140 mM potassium glutamate, 5 mM glucose, and 5 mM EGTA). After permeabilization, the medium was replaced subsequently with 250 μl KGEP containing 10 μM eserine (Sigma) with or without various amounts of $CaCl_2$. Ca^{2+} concentrations in buffers were calculated according to Nanninga and Kempen (27). When the effects of Mg and ATP (Yamasa, Chiba, Japan) or guanosine 5′-(γ-thio)triphosphate (GTPγS, Boehringer Mannheim) were examined, the Ca^{2+}-stimulus incubation was done in the presence of the drug. At the end of the incubation periods, the buffer was immediately transferred to a microtube containing 25 μl 2 M percholic acid (PCA). Cells were sonicated on ice with 500 μl chilled 0.2 M PCA. The samples were centrifuged at 15,000 $\times g_{av}$ for 5 min at 4°C and the supernatant was stored at −80°C until the assay of ACh and CA.

Secretion of ACh and CA from PC12 cells treated with a phorbol ester. The treatment of PC12-C3 cells with phorbol myristate acetate (PMA) was carried out as described previously (28). The cells were then washed with the standard assay buffer (50 mM Hepes-Tris buffer, pH 7.4 containing 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5.5 mM glucose) and stimulated by elevating the potassium concentration from 5 to 30 mM as described (24). Released and cellular ACh and CA were extracted with 0.2 M PCA as described above.

ACh assay by HPLC. To determine ACh content, 5 μl of 50% (v/v) saturated solution of K_2CO_3 was added to 150 μl of the samples for neutralization. After a 30 min incubation on ice, the samples were centrifuged at 15,000 $\times g_{av}$ for 5 min at 4°C and the supernatants were filtered through a 0.22-μm pore membrane filter (Ultrafree-C3-LG, Millipore) after the addition of 15 μl of 50 mM EDTA. Aliquots (10-25 μl) were injected into HPLC with an electrochemical detector (ECD) system.

The HPLC-ECD procedure was developed originally by Potter *et al.* (29) and improved by Asano *et al.* (30). The system consists of a reverse-phase column (Eicompak AC-GEL, 6 mm × 15 cm, Eicom), an immobilized acetylcholine esterase and choline oxidase column (AC-Enzympak, 4 mm × 5 mm, Eicom), and an ECD (ECD-300, Eicom) with a platinum electrode. The mobile phase was composed of 0.1 M Na_2HPO_4 buffer (pH 8.5) containing 20 μM EDTA, 65 mg/L tetramethylammonium chloride (Sigma), and 200 mg/L sodium 1-decanesulfonate (Tokyo Kasei Kogyo, Tokyo, Japan). The columns were maintained at 33°C with a column oven and at a flow rate of 1 ml/min. The applied potential at the working electrode was +450 mV versus the Ag/AgCl reference electrode and the detector range at full scale was 0.1 nA. The amount of ACh released is expressed as a percentage of total cellular ACh content.

CA assay by HPLC. Released and cellular CA were assayed by HPLC using a reverse-phase column (TSKgel ODS-80T_M, 4.6 mm × 10 cm, Tosoh, Tokyo, Japan) and an ECD as described (31). The mobile phase was composed of 85 mM NaH_2PO_4 buffer (pH 3.7) containing 15% (v/v) methanol, 20 μM EDTA, and 2.5 mM sodium 1-octanesulfonate (Nacalai). The column was maintained at 34°C and the flow rate was 1 ml/min. The applied potential at the working electrode was at +700 mV against Ag/AgCl and the detector range was 2 nA full scale. Essentially, the only CA detected in PC12-C3 cells was dopamine. The amount of CA released is expressed as a percentage of the total cellular CA content.

RESULTS AND DISCUSSION

Because most of the PC12 cells which we have obtained from several sources have almost lost the ability to synthesize ACh, we isolated a subclone of PC12 (PC12-C3) which stores a high content of acetylcholine (~380 pmol per 10^6 cells), by single cell recloning (Y. Shoji-Kasai, manuscript in preparation). PC12-C3 cells released ACh in response to high-K^+-depolarization only in the presence of Ca^{2+} in the extracellular medium (data not shown).

PC12-C3 cells were permeabilized by an incubation with 8 μM digitonin and were stimulated with various concentrations of Ca^{2+}. ACh released into the extracel-

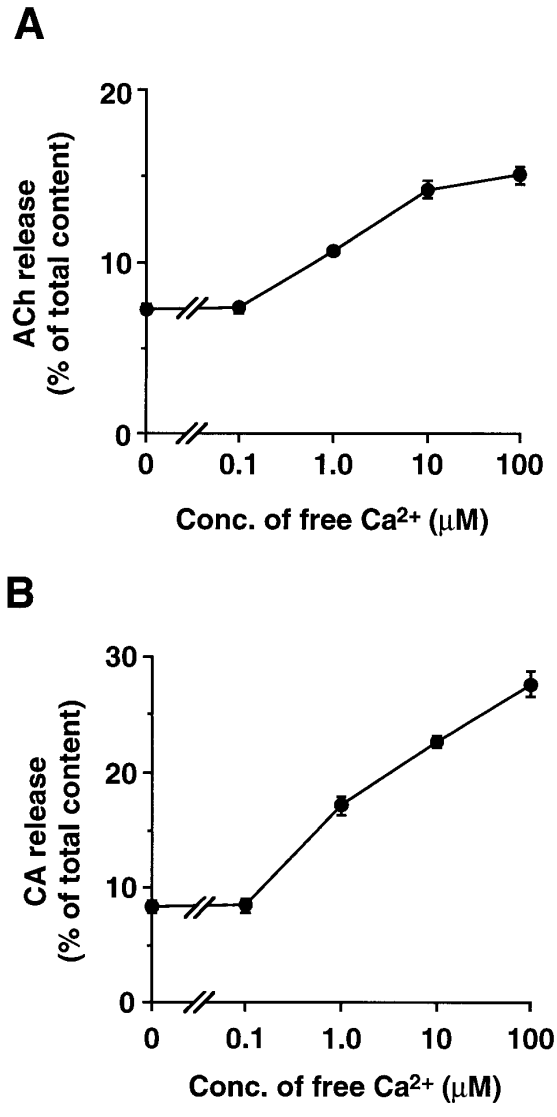

FIG. 1. Ca^{2+}-dependency of ACh (A) and CA (B) release from digitonin-permeabilized PC12-C3 cells. Cells were permeabilized for 5 min with 8 μM digitonin in KGEP in the presence of 5 mM MgATP. The cells were subsequently incubated with KGEP containing various concentrations of Ca^{2+} in the absence of MgATP. Transmitter release was determined after 6 min. The amounts of ACh and CA released are expressed as a percentage of the total cellular content. The values are the means ± S.E.M. from four representative experiments.

lular medium was measured simultaneously with CA using the HPLC-ECD system. ACh release was observed when the Ca^{2+} concentration in the medium was elevated above 1 μM and the amount of released ACh increased with increasing Ca^{2+} concentrations up to 100 μM (Fig. 1A). The release of CA in digitonin-permeabilized cells displayed a similar Ca^{2+}-dependency to ACh release (Fig. 1B). Physiological studies show that the relative proportion of the exocytosis of the contents of LDCVs and SSVs from the same nerve endings varies with the frequency of stimulation (32). A higher frequency of stimulation is necessary to induce the fusion of LDCVs than that required for the fusion of SSVs, suggesting that the topological relationship to Ca^{2+} channels, or the sensitivity to cytosolic Ca^{2+} may be different between these two types of vesicle. Present findings indicate that there is not a significant difference in the Ca^{2+} sensitivities between SLMVs and secretory vesicles, and that topological considerations are more likely to be involved in determining dependency on stimulation frequency.

It has been well established that Ca^{2+}-induced CA release comprises at least two sequential steps: a MgATP-dependent priming step and a MgATP-independent Ca^{2+}-triggered fusion step (7). When permeabilized adrenal chromaffin cells are challenged only by Ca^{2+} in the absence of MgATP, the rate of CA release is rapid and terminates within 2 min. This MgATP-independent release is considered to reflect the exocytotic response of the already primed secretory vesicles by endogenous MgATP before permeabilization. In contrast, CA release evoked by Ca^{2+} in the presence of MgATP occurs at a slower rate than MgATP-independent release and continues at a relatively constant rate for 12 min. MgATP-dependent Ca^{2+}-evoked release seems to reflect the Ca^{2+}-triggered exocytosis of additional secretory vesicles primed by exogenous MgATP. Similar steps in the exocytosis of secretory vesicles have also been observed in permeabilized PC12 cells (Ref. 8 and Fig. 2B). In order to know whether the exocytosis of SLMVs involves a MgATP-dependent priming step, we next examined the time course of Ca^{2+}-dependent ACh release from the permeabilized PC12-C3 cells in the presence and absence of exogenous MgATP. Ca^{2+}-dependent release of ACh in the presence and absence of MgATP was similar during the first minute (Fig. 2A). ACh release ended within 2-4 min in the absence of MgATP but continued for at least 6 min in its presence. These results indicate that the MgATP-priming step is also necessary for the exocytosis of SLMVs.

Many reports have shown that guanine nucleotides modify CA release from adrenal chromaffin and PC12 cells (26, 33-38). Figure 3 shows the effects of the non-hydrolyzable analogue, GTPγS, on the Ca^{2+}-dependent and independent release of ACh and CA from digitonin-permeabilized PC12-C3 cells. GTPγS (100 μM) caused about a twofold increase in the amount of ACh and CA released during a 5-min incubation in the absence of Ca^{2+}. ACh and CA release induced by GTPγS was about 30 and 20% of that induced by 10 μM Ca^{2+}, respectively. Simultaneous administration of GTPγS with 10 μM Ca^{2+} did not give an increase of the release of either ACh or CA over that measured with Ca^{2+} alone, suggesting that GTPγS uses the same exocytotic machinery as Ca^{2+}. At the frog neuromuscular junction, complete dissociation between the exocytosis of

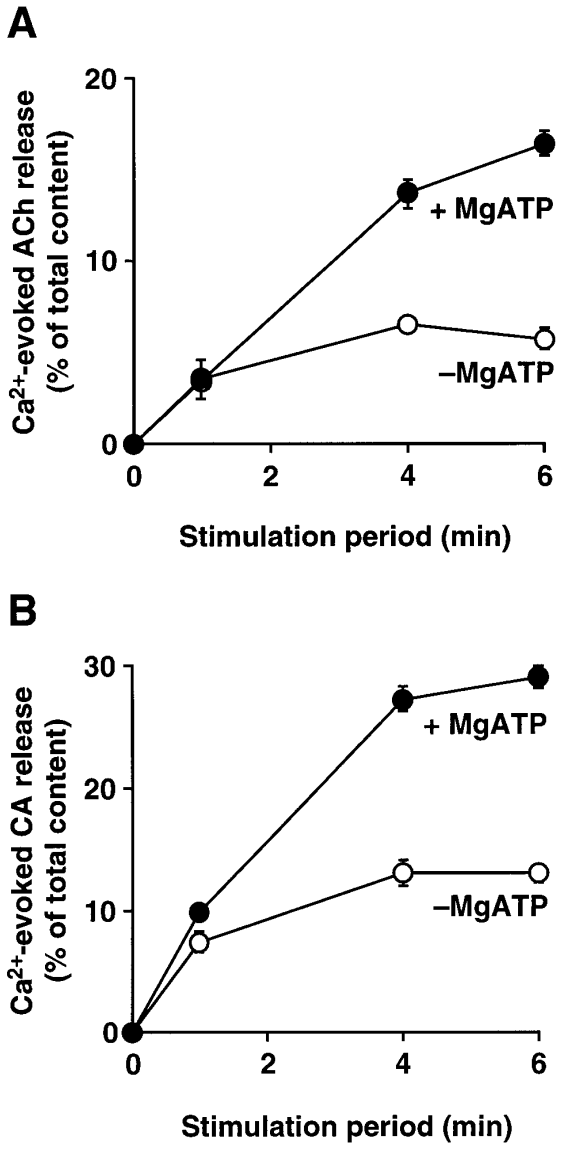

FIG. 2. Time course of ACh (A) and CA (B) release from permeabilized PC12-C3 cells in the presence and absence of MgATP. Cells were permeabilized in the absence of MgATP. After 5 min the solution was replaced with KGEP with or without 10 μM Ca^{2+} in the presence (closed circle) or absence (open circle) of 5 mM MgATP. ACh and Ca release was measured at the indicated times after the change of solution. Ca^{2+}-evoked release was calculated by subtracting the release in the absence of Ca^{2+} from that in the presence of Ca^{2+}. The values are the means ± S.E.M. from four representative experiments.

SSVs and LDCVs is observed. When α-latrotoxin, a protein toxin from black widow spider venom, is applied in the absence of extracellular Ca^{2+}, it induces a rapid depletion of ACh-containing SSVs without producing any significant decrease in the number of LDCVs containing calcitonin gene-related peptide in the same nerve endings (39). Recently, a Ca^{2+}-independent receptor of α-latrotoxin has been identified as a novel orphan GTP binding protein-coupled receptor (40). These findings raise the possibility that SSVs and LDCVs are differentially regulated by GTP binding proteins. However, the present study shows that at least in PC12 cells, there is no significant difference in the effects of guanine nucleotides on ACh and CA release, suggesting that exocytosis from SLMVs and secretory vesicles are regulated by GTP binding proteins in a similar way.

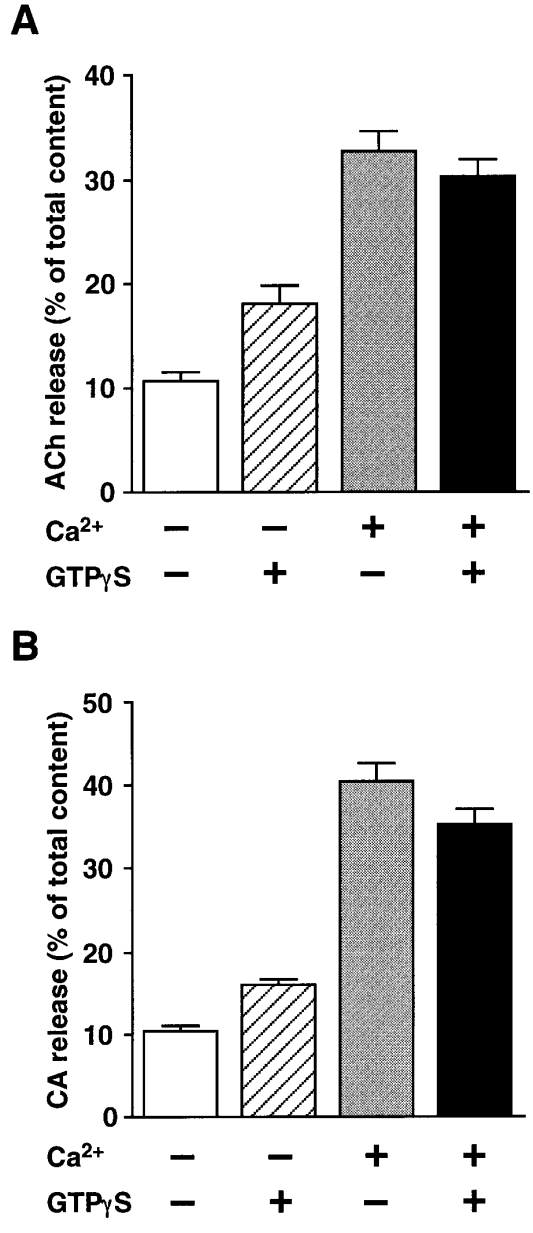

FIG. 3. Effects of GTPγS on ACh (A) and CA (B) release from permeabilized cells. PC12-C3 cells were permeabilized for 5 min with digitonin in KGEP containing 5 mM MgATP and then incubated for 5 min in the presence of 5 mM EGTA (open bar), 100 μM GTPγS (hatched bar), 10 μM Ca^{2+} (gray bar), or GTPγS with 10 μM Ca^{2+} (black bar) in KGEP containing 5 mM MgATP. The values are the means ± S.E.M. from four representative experiments.

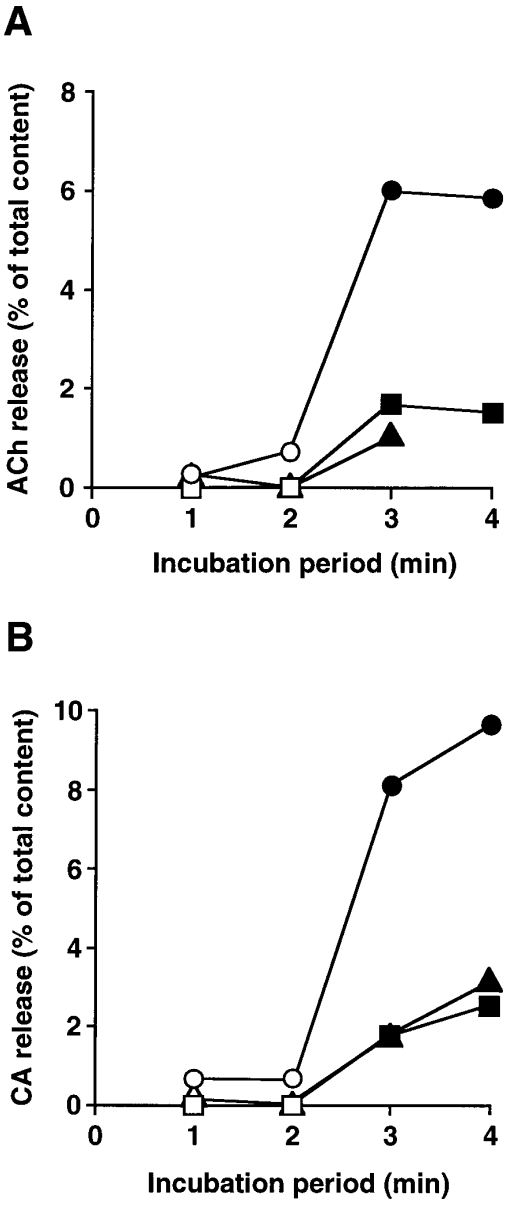

FIG. 4. Effects of phorbol esters on high-K^+-induced ACh (A) and CA (B) release from intact PC12-C3 cells. Intact PC12-C3 cells were incubated for 20 min in the presence or absence (squares) of 0.1 μM phorbol ester [either PMA (circles) or 4α-phorbol (triangles)]. Then, the extracellular medium was changed four times every minute, and the amount of ACh and CA released in the media was determined and expressed as a percentages of the total cellular contents. The K^+ concentration of the third and fourth media was elevated from 5 to 30 mM to depolarize the cells (closed symbols).

Involvement of protein kinase C in the regulation of CA release from PC12 cells is demonstrated by the finding that phorbol esters, which activate protein kinase C, can potentiate secretory responses (25, 41). As shown in Fig. 4, treatment with an active phorbol ester PMA markedly enhanced high-K^+-dependent release of both ACh and CA from intact PC12-C3 cells. Such enhancement was not induced by a phorbol, 4α-phorbol, which does not activate protein kinase C. These results suggest that exocytosis of the contents of SLMVs and secretory vesicles are both regulated by protein kinase C-mediated phosphorylation.

In summary, we have succeeded in measuring the exocytotic activity of SLMVs and secretory vesicles simultaneously from PC12 cells. Our findings indicate that these two types of vesicle share many common mechanisms for exocytosis.

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