Calmodulin and Protein Kinase C Increase Ca\(^{2+}\)-stimulated Secretion by Modulating Membrane-attached Exocytic Machinery*

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The molecular mechanisms underlying the Ca\(^{2+}\) regulation of hormone and neurotransmitter release are largely unknown. Using a reconstituted \([3H]norepinephrine release assay in permeabilized PC12 cells, we found that essential proteins that support the triggering stage of Ca\(^{2+}\)-stimulated exocytosis are enriched in an EGTA extract of brain membranes. Fractionation of this extract allowed purification of two factors that stimulate secretion in the absence of any other cytosolic proteins. These are calmodulin and protein kinase Cα (PKCα). Their effects on secretion were confirmed using commercial and recombinant proteins. Calmodulin enhances secretion in the absence of ATP, whereas PKC requires ATP to increase secretion, suggesting that phosphorylation is involved in PKC- but not calmodulin-mediated stimulation. Both proteins modulate release events that occur in the triggering stage of exocytosis. The half-maximal increase was elicited by 3 nM PKC and 75 nM calmodulin. These results suggest that calmodulin and PKC increase Ca\(^{2+}\)-activated exocytosis by directly modulating the membrane- or cytoskeleton-attached exocytic machinery downstream of Ca\(^{2+}\) elevation.

The molecular mechanisms of presynaptic vesicle release have been extensively examined by a combination of biochemical, genetic, and electrophysiological techniques. A series of protein-protein interaction cascades have been proposed to lead to vesicle docking and fusion (1–3). The SNARE protein family, including syntaxin, SNAP-25, and vesicle-associated membrane protein (VAMP), also called synaptobrevin, plays an essential role in promoting membrane fusion, and is thought to comprise the basic fusion machinery (4, 5). In Ca\(^{2+}\)-stimulated exocytosis, many additional proteins are important in the Ca\(^{2+}\) regulation of the basic membrane trafficking apparatus. Calcium not only triggers rapid fusion of release-competent vesicles, but is also involved in earlier processes which replenish the pool of readily releasable vesicles (6). Furthermore, it appears to be critical in initiating several forms of synaptic plasticity including post-tetanic potentiation (7). The molecular mechanisms by which Ca\(^{2+}\) regulates these processes is not well understood.

PC12 cells have often been utilized to study Ca\(^{2+}\)-activated exocytosis, as they offer a homogeneous cell population that possesses the same basic exocytic machinery as neurons (8). In this study, we used an established cracked cell assay, in which \([3H]norepinephrine (NE)\) labeled PC12 cells are permeabilized by mechanical “cracking” and then reconstituted for secretion of NE in the presence of test proteins (9). Transmitter-filled vesicles and intracellular cytoskeletal structures remain intact in these cells, while cytosolic proteins leak out (10). These cracked cells readily release NE upon addition of ATP, brain cytosol, and 1 μM free Ca\(^{2+}\) at an elevated temperature. We term this a “composite assay,” as all essential components are added into one reaction mixture. Alternatively, cracked cells can be first primed with cytosol and ATP, washed, then reconstituted for NE release with cytosol and Ca\(^{2+}\) (11). This sequential priming-triggering protocol is useful for determining whether a protein acts early or late in the exocytic pathway and whether its effect is dependent on Ca\(^{2+}\) or ATP.

This semi-intact cell system serves as a bridge between an in vitro system comprised of purified components and electrophysiological systems that monitor release in vivo. It provides information on protein functions in a cell with an intact membrane infrastructure while being easily manipulable. Ca\(^{2+}\) regulation by membrane depolarization is no longer a concern, as intracellular Ca\(^{2+}\) concentration can be controlled by a buffered solution. Indirect readout of neurotransmitter release using a postsynaptic cell is replaced by direct readout of \([3H]NE\) released into the buffer. Complications associated with interpreting overlapping exo- and endocytotic signals are also eliminated as only one round of exocytosis is measured. Finally, concentration estimates are likely to be accurate, since added compounds do not need to diffuse long distances along axons and dendrites to their sites of action.

Using this assay, several proteins required for NE release have been purified from rat brain cytosol, including phosphatidylinositol transfer protein (12), phosphatidylinositol-4-phosphate 5-kinase (13), and calcium-dependent activator protein for secretion (CAPS) (9). The validity of the cracked cell system is confirmed by the finding that phosphatidylinositol transfer protein and CAPS are mammalian homologues of yeast SEC14p (12) and nematode UNC31p, respectively (14), both proteins involved in membrane trafficking (15, 16).

Calmodulin is the most ubiquitous calcium mediator in eu-

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The abbreviations used were: NE, norepinephrine; PKC, protein kinase C; CaM, calmodulin; SNAP-25, synaptosome-associated protein of 25 kDa; CAPS, calcium-dependent activator protein for secretion; SNAP, SNAP (soluble N-ethylmaleimide-sensitive factor attachment proteins) receptor; CaMK, Ca\(^{2+}\)/calmodulin-dependent protein kinase; PAGE, polyacrylamide gel electrophoresis; AMP-PNP, adenosine 5’-(β,γ-imido)triphosphate; HA, hydroxyapatite.
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karyotic cells, yet its involvement in membrane trafficking has not been well established. Some early studies showed that calmodulin inhibitors (17–19), anti-calmodulin antibodies (20, 21), or calmodulin binding inhibitory peptides (22) inhibited Ca$^{2+}$-activated exocytosis. However, in other studies, calmodulin-binding peptides and an anti-calmodulin antibody led to the conclusion that calmodulin is only involved in endocytosis, not exocytosis (23). More recently, it was reported that Ca$^{2+}$/calmodulin signals the completion of docking and triggers a late step of homotypic vacuole fusion in yeast, thus suggesting an essential role for Ca$^{2+}$/calmodulin in constitutive intracellular membrane fusion (24). If calmodulin indeed plays an important role in exocytosis, a likely target of calmodulin is Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMkII), a multifunctional kinase that is found on synaptic vesicles (25) and has been shown to potentiate neurotransmitter release (26, 27).

Another Ca$^{2+}$ signaling molecule, PKC, has also been implicated in regulated exocytosis. In various cell systems, it has been shown that the phorbol esters stimulate secretion (28, 29). It is usually assumed that phorbol esters affect exocytosis by a signaling mechanism that can be activated by the PKC pathway (30, 31), which complicates the interpretation of some earlier reports. The mode of action of PKC remains controversial. There is evidence that PKC increases the intracellular Ca$^{2+}$ levels by modulating plasma membrane Ca$^{2+}$ channels (32, 33), that it increases the size of the release-competent vesicle pool (34, 35), or that it increases the Ca$^{2+}$ sensitivity of the membrane trafficking apparatus (36). However, no consensus on these issues has been reached.

PKC substrates that have been implicated in exocytosis include SNAP-25 (37), syntaptotagmin (28), CAPS (38), and n-secl (39). It is believed that upon phosphorylation, these PKC substrates might interact differently with their binding partners, which, in turn, leads to the enhancement of exocytosis. In addition, evidence is accumulating that PKC and calmodulin interfere with each other's actions, as PKC phosphorylation sites are embedded in the calmodulin-binding domains of substrates such as neuromedin and neurogranin (40). It is therefore possible that PKC could modulate exocytosis via a calmodulin-dependent pathway by synchronously releasing calmodulin from storage proteins.

In this study, we fractionated an EGTA extract of brain membranes in order to identify active components that could reconstitute release in the cracked cell assay system. We identified calmodulin and PKC as two active factors. Thus, we demonstrate that calmodulin and PKC play a role in the Ca$^{2+}$ regulation of exocytosis, and provide further insight into the mechanisms of their action.

**EXPERIMENTAL PROCEDURES**

**Materials**

Bisindolylmaleimide I, PKC inhibitor peptide (19–36), human recombinant PKCs, rat brain PKC, and anti-PKC monoclonal antibody (Ab2) were purchased from Calbiochem (San Diego, CA). Calmodulin from bovine brain was obtained from Sigma, annexin VI (annexin 67 kDa) from bovine liver was obtained from Sigma and Biodigios (Kennebunk, ME), and anti-calmodulin monoclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Calmodulin-binding peptide (CaMkII an 291–312) was synthesized at Research Genetics (Huntsville, AL) and high pressure liquid chromatography purified.

**Rat Brain Cytosol Preparation**

Frozen rat brains (Harlan, IN) were homogenized in 3 ml/g of ice-cold homogenization buffer (20 mM Hepes, pH 7.5, 0.25 mM sucrose, 2 mM EGTA, 2 mM EDTA, 2 µM leupeptin, 4 µM aprotinin, 1 mM diithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) using a VirTis homogenizer (Virtis, NY). The homogenate was centrifuged at 30,000 x g for 20 min, and the supernatant was centrifuged again at 100,000 x g for 1 h. The resulting supernatant was rapidly frozen in aliquots and stored at −80 °C.

**Membrane EGTA Extract Preparation**

Frozen rat or bovine brains (RJO Biologicals, Kansas City, MO) were homogenized as above, except that the buffer lacks 2 mM EGTA and 2 mM EDTA (1 liter/bovine brain). The homogenate was centrifuged twice at 3,000 x g for 15 min, and the supernatant centrifuged at 100,000 x g for 1 h. The 100,000 x g supernatant, if kept, was rapidly frozen and stored as cytosol. It is referred to in the text as "cytosol prepared in the absence of EGTA." The pellet of the 100,000 x g spin was washed once with a homogenization buffer without protease inhibitors, using a Teflon/glass homogenizer, and centrifuged at 100,000 x g for 1 h. The washed membrane pellet was then homogenized into a homogenization buffer without protease inhibitors at about 0.5 ml/g of starting material. EGTA was added to reach 2 mM final concentration, and the extraction was carried out at 4 °C for a few hours or overnight. The membrane homogenate was then centrifuged at 100,000 x g for 1 h, and the supernatant collected as "membrane EGTA extract." The protein concentration of cytosol and membrane EGTA extract was estimated using a Bradford protein assay (Bio-Rad), using bovine serum albumin as a standard. In a typical preparation, the protein concentration of rat brain cytosol ranges from 2 to 9 mg/ml, and the membrane EGTA extract 0.3 to 0.6 mg/ml. About 3% of total membrane proteins can be extracted into the EGTA extract.

**Cracked Cell Assay**

PC12 cells were maintained and [3H]NE labeled as described previously (11). Labeled cells were harvested by pipetting with ice-cold potassium glutamate buffer (50 mM Hepes, pH 7.2, 105 mM potassium glutamate, 2 mM potassium acetate, 2 mM EGTA) containing 0.1% bovine serum albumin. All subsequent manipulations were carried out at 0–4 °C unless otherwise stated. Labeled cells (1–1.5 ml/dish) were mechanically permeabilized by a single passage through a chilled stainless steel ball homogenizer. The cracked cells were adjusted to 11 mM EGTA and incubated on ice for 0.5–3 h, followed by three washes in which the cells were centrifuged at 800 x g for 5 min and resuspended in potassium glutamate buffer containing 0.1% bovine serum albumin. 

**Composite Assay—** Each release reaction contains 0.5–1 million cracked cells, 1.5 µM free Ca$^{2+}$, 2 mM MgATP, and the protein solution to be tested in a total volume of 200 µl of potassium glutamate buffer. Release reactions were initiated by incubation at 30 °C for 0.5–1 min and terminated by returning to ice after a 15-min incubation. The supernatant of each reaction was assayed for Ca$^{2+}$ release by centrifugation at 2,500 x g for 15 min, followed by three washes in which the cells were centrifuged at 8,000 x g for 5 min and resuspended in potassium glutamate buffer containing 0.1% bovine serum albumin. 

**Concentration Determination—** Each release reaction contains 0.5–1 million cracked cells, 1.5 µM free Ca$^{2+}$, 2 mM MgATP, and the protein solution to be tested in a total volume of 200 µl of potassium glutamate buffer. Release reactions were initiated by incubation at 30 °C for 0.5–1 min and terminated by returning to ice after a 15-min incubation. The supernatant of each reaction was assayed for Ca$^{2+}$ release by centrifugation at 2,500 x g for 15 min, followed by three washes in which the cells were centrifuged at 8,000 x g for 5 min and resuspended in potassium glutamate buffer containing 0.1% bovine serum albumin. 

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**Composite Assay—** Each release reaction contains 0.5–1 million cracked cells, 1.5 µM free Ca$^{2+}$, 2 mM MgATP, and the protein solution to be tested in a total volume of 200 µl of potassium glutamate buffer. Release reactions were initiated by incubation at 30 °C and terminated by returning to ice after a 15-min incubation. The supernatant of each reaction was assayed for Ca$^{2+}$ release by centrifugation at 2,500 x g for 30 min at 4 °C, and the released [3H]NE was quantified by scintillation counting (Beckman LS6000IC). Cell pellets were dissolved in 1% Triton X-100, 0.02% azide and similarly counted. NE release was calculated as a percentage of total [3H]NE in the supernatant.

**Priming Assay—** A priming reaction contains about 1–2 million cracked cells, 2 mM MgATP, and the protein solution to be tested in a total volume of 200 µl of potassium glutamate buffer. Note that Ca$^{2+}$ is omitted. The reaction was carried out at 30 °C for 30 min. The primed cells were then spun down, washed once with fresh potassium glutamate buffer, and distributed into two triggering reactions, each containing 10 µl of rat brain cytosol and 1.5 µM free Ca$^{2+}$ in a total volume of 200 µl of potassium glutamate buffer. The triggering reaction was performed at 30 °C for 3 min, and the NE release was measured as a composite assay.

**Free Ca$^{2+}$ Concentration Determination**

The range of [Ca$^{2+}$]free in the release reaction (Fig. 2B) was achieved by adding Ca$^{2+}$ into potassium glutamate buffer to reach final [Ca$^{2+}$]free in the range of 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 1.9, and 2.0 mM. The pH of the reaction was 7.24 when no Ca$^{2+}$ was added and 7.04 when 2.0 mM Ca$^{2+}$ was added, in the absence of protein extracts or cracked cells. Free Ca$^{2+}$ concentrations were determined using video microscopic measure-
ments of fura-2 fluorescence. Imaging was performed as described previously (41). \( \frac{[\text{Ca}^{2+}]_{\text{free}}}{[\text{Ca}^{2+}]_{\text{total}}} \) was calculated from the equation \( [\text{Ca}^{2+}]_{\text{free}} = \frac{K_{\text{d}} \times (R - R_{\text{min}}) \times (R_{\text{max}} - R)}{K_{\text{d}} + K_{\text{d}}^{\ast}} \). The values of \( R_{\text{min}}, R_{\text{max}}, \) and \( K_{\text{d}}^{\ast} \) were determined in the following solutions: 1) \( R_{\text{min}} \): potassium glutamate buffer containing \( 8 \times 10^6 \) cracked cells/ml, \( 2 \text{ mM MgATP, and } 10 \text{ mM total Ca}^{2+}; 2) R_{\text{max}} \): potassium glutamate buffer containing \( 8 \times 10^6 \) cracked cells/ml, \( 2 \text{ mM MgATP, and } 10 \text{ mM total Ca}^{2+}; 3) K_{\text{d}}^{\ast} \): potassium glutamate buffer containing \( 8 \times 10^6 \) cracked cells/ml, \( 2 \text{ mM MgATP, and } 10 \text{ mM total Ca}^{2+}. \)

Purification of Active Proteins

All procedures were carried out at 4 °C or on ice unless noted otherwise. Membrane EGTA extract of one or two bovine brain(s) (usually 150 ml/brain) was filtered through cheesecloth and loaded overnight onto a 10- or 20-ml DEAE column packed with DEAE-Sepharose CL-6B beads (Amersham Pharmacia Biotech). The column was washed with wash buffer (20 mM Hepes, pH 7.5, 0.25 mM sucrose, 2 mM EGTA, 1 mM dithiothreitol) and step eluted with 10 column volumes of elution buffer (20 mM Hepes, pH 7.5, 2 mM EGTA, 400 mM KCl, 1 mM dithiothreitol), 100 \( \mu \text{M} \) of every other fraction was dialyzed overnight into potassium glutamate buffer, and 20 \( \mu \text{M} \) tested in a composite release assay for activity. The active fractions were pooled and dialyzed into zero salt buffer (20 mM Hepes, pH 7.5, 2 mM EGTA) and batch bound to 10 ml of Affi-Gel Blue beads (Bio-Rad) or DyeMatrix-Green A beads (Amicon) for a few hours. Blue beads were used in earlier experiments, and Green beads were used later to specifically depleted CAPS, which was known to bind to Green beads (9). The unbound material was collected, concentrated to about 2 ml using a Centriprep-10 (Amicon), and loaded onto a 120-ml HiPrep Sephacryl S-200 gel filtration column (Amersham Pharmacia Biotech). Samples were run on the S-200 column in potassium glutamate buffer at a flow rate of 7 ml/h. 10–50 \( \mu \text{l} \) of every other fraction was tested for activity in the cracked cell composite assay, and two peaks of activity were observed (Fig. 3).

The first peak of activity had a predicted molecular mass of 85 kDa. The corresponding material was adjusted to 10 mM potassium phosphate concentration (pH 7.2) and loaded onto a 1-ml hydroxyapatite column packed with hydroxyapatite Bio-Gel HT (Bio-Rad). The bound material was eluted with a linear potassium phosphate gradient from 10 to 500 mM (pH 7.2) in a 30-ml total volume at a flow rate of about 0.1 ml/min. The first four fractions were collected; 200 \( \mu \text{l} \) of each fraction was dialyzed into potassium glutamate buffer and 20 \( \mu \text{l} \) tested for activity. Meanwhile, the fractions (10 \( \mu \text{l} \) each) were also analyzed by SDS-PAGE and silver staining (Sigma silver stain kit). The active material was concentrated and resolved on an 8% polyacrylamide gel. Two Coomassie-stained protein bands that matched the activity profile (Fig. 6) were excised from the gel, minced into small pieces, dried, and sequenced by the Stanford PAN facility. The two polypeptide sequences obtained from the upper band were: LLIQEEGYYVNPIXEGD and IRSTLNPRDWDEST. The only bovine protein that contains both polypeptides is PKCa. The four polypeptide sequences obtained from the lower band were: YELTGKFERLVLGMRPAFY, LEIILASRTNE-QHQLVAA, MLVLLQGTREDDVSESDE, and EMSGDVRDVFV-AIVQSVK. Based on these sequences, the protein band was unambiguously identified to be bovine annexin VI.

The second 200-peak has a predicted molecular mass of 25 kDa. The corresponding material was dialyzed into zero salt buffer (20 mM Tris, pH 7.5, 1 mM EGTA) and injected onto a Mono-Q HR 5/5 FPLC column (Pharmacia). The FPLC run was performed at 18 °C at 1 ml/min and 1-ml fractions were collected with a linear salt gradient from 0 to 1 mM KCl. The 1-ml fractions containing proteins (determined by \( A_{280} \)) were dialyzed into potassium glutamate buffer and tested in the cracked cell assay.

Western Blot

Anti-calmodulin antibody and anti-PKCa antibody were used at 1:2000 and 1:100 dilution at room temperature for 1 h. ECL (Amersham) was used for detection.

RESULTS

A Membrane EGTA Extract Supports NE Release—Brain cytosol, prepared as the supernatant of a 100,000 \( \times g \) centrifugation of the brain homogenate, effectively stimulates NE release in the cracked cell assay (Fig. 1) as previously shown (9). We wondered whether crude extracts other than cytosol could also support NE release, and we focused our attention on extractable peripheral membrane proteins. We found that a salt or EGTA extract of brain membranes, membranes defined as the 100,000 \( \times g \) pellet of the crude homogenate, reconstituted secretion in the absence of cytosol. Whereas the salt extract only slightly enhanced NE release above background (data not shown), the EGTA extract not only stimulated NE release to a high level, similar to that supported by cytosol, but also had a higher specific activity than cytosol (Fig. 1). The ability of the membrane EGTA extract to support secretion is consistent with the fact that following cracking, the cells are immediately extracted with EGTA, and are presumably devoid of most membrane EGTA-extractable factors. This also suggests that these factors, some of which are probably \( \text{Ca}^{2+} \)-dependent membrane-associating proteins, participate in \( \text{Ca}^{2+} \)-triggered exocytosis.

The Membrane EGTA Extract Is Enriched in Triggering Factors—NE release in cracked cells can be resolved into two sequential stages, an ATP-dependent priming stage and an ATP-independent \( \text{Ca}^{2+} \)-dependent triggering stage (11), and proteins can be tested for activity in either stage. An effect in priming indicates an early role for the protein, and an effect in triggering a late ATP-independent role. Since the protein composition of the membrane EGTA extract and cytosol are different, we tested whether they had different activities in the priming stage versus the triggering stage. We found that the membrane EGTA extract is enriched in factors that act during triggering stage of NE release, as the same amount of protein from the membrane EGTA extract as cytosol gave a higher stimulation in the triggering assay, but not in the priming assay (Fig. 2A). Regular cytosol is prepared in a buffer containing 2 mM EGTA, and thus presumably contains some of the proteins present in the membrane EGTA extract. Cytosol prepared in the absence of EGTA showed an even lower specific activity in the triggering assay compared with regular cytosol (Fig. 2A).

Another way to assay whether a protein is involved in priming or triggering is to analyze the \( \text{Ca}^{2+} \) sensitivity of its effect. If the cells were not primed with ATP, stimulation of NE release occurred over a relatively wide range of \( \text{Ca}^{2+} \) concentrations with the addition of cytosol (Fig. 2B, left panel). How-
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\section*{Fig. 2. The membrane EGTA extract is enriched in triggering factors.} 
\textbf{A}, rat brain membrane EGTA extract (closed triangles), rat brain cytosol (closed squares), and rat brain cytosol prepared in the absence of EGTA (open triangles) were tested for their stimulatory effects either in the priming (left) or triggering (right) stage of NE release. \textbf{B}, NE release was measured with varying concentrations of free Ca\textsuperscript{2+} either in a composite assay (left) or triggering assay (right). Addition of 10 \mu{g} of rat brain membrane EGTA extract (closed triangles) or 40 \mu{g} of rat brain cytosol (closed squares) increased NE release as compared with conditions in which no proteins were added (open squares).

However, if the cells were primed with ATP before the addition of cytosol, stimulation was only observed at relatively high levels of Ca\textsuperscript{2+} (Fig. 2B, right panel). Therefore, priming seems to correspond to an increase in release at low [Ca\textsuperscript{2+}], and triggering corresponds to release at high [Ca\textsuperscript{2+}]. In contrast to cytosol, membrane EGTA extract preferentially stimulated release at high [Ca\textsuperscript{2+}] (Fig. 2B), suggesting again that the membrane extract is enriched in triggering factors.

\section*{Identification of Calmodulin as an Active Triggering Factor in the EGTA Extract—Biochemical fractionation of the bovine brain membrane EGTA extract was carried out to identify the active components capable of reconstituting NE release. Activity was assayed in a composite reaction mixture containing cracked cells, ATP, Ca\textsuperscript{2+}, and the test protein(s). Note that, except for the presence of bovine serum albumin in the basal buffer, no other proteins were added to the cell ghosts except for the test protein(s). Initial tests indicated that at least part of the activity in the membrane EGTA extract binds to and can be efficiently eluted from an anion exchanger and hydroxyapatite resin, but does not bind to Amicon color resins. The starting material was, therefore, sequentially purified using DEAE, Affi-Gel Blue (or Matrex Green-A), and gel filtration chromatography. Gel filtration chromatography indicated the presence of two peaks of activity with predicted molecular masses of 25 and 85 kDa, respectively (Fig. 3). The low molecular weight active factor was purified to homogeneity, as judged by a Coomassie-stained SDS-PAGE gel, after a subsequent Mono-Q fractionation (Fig. 4).

We reasoned that the protein might be calmodulin (43) based on the following observations. 1) It is a relatively small protein (14–18 kDa) that is abundant in the starting extract (Fig. 4A). 2) It elutes at a very high salt concentration (0.4 M KCl) on the Mono-Q column. 3) It stains negatively in silver stain (data not shown). 4) Its electrophoretic mobility shifts depending on the presence or absence of Ca\textsuperscript{2+} (Fig. 4C). A Western blot with an anti-calmodulin monoclonal antibody gave a positive signal (Fig. 4D), confirming our prediction.

\section*{Properties of Calmodulin-stimulated Exocytosis—We used commercial calmodulin or bacterially expressed recombinant calmodulin to confirm our purification result; both sources of authentic calmodulin stimulated NE release as expected. Moreover, we found that calmodulin stimulates secretion in a trig-
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Calmodulin stimulates NE release in the triggering stage. A, calmodulin (obtained from Sigma) increased NE release in the triggering assay in a dose-dependent fashion, in the absence of ATP or any other cytosolic proteins. In this particular experiment, the maximal release achieved by addition of rat brain cytosol was 46.5%. B, the triggering assay was performed with different concentrations of free Ca\(^{2+}\). Calmodulin (3 \(\mu g\) bacterially expressed recombinant protein; closed squares) increased NE release with a similar Ca\(^{2+}\) sensitivity to rat brain membrane EGTA extract (10 \(\mu g\); closed triangles), as compared with conditions in which no protein was added (open squares).

Western analysis with commercial protein as standards in SDS-PAGE. The first three lanes were visualized by Coomassie Blue staining. The fourth lane and the gel showing HA fractions (10 \(\mu l\) fraction) were visualized by silver staining. The two arrowheads indicate the two bands that were identified in panel C as PKCs and annexin VI, B, the activity of HA fractions 12–25 in a composite release assay (20 \(\mu l\) fraction). C, the active HA fractions (fraction 16–21), were pooled, concentrated, and resolved on an 8% SDS-PAGE gel. The two candidate Coomassie bands corresponding to the activity (arrowheads) were excised and sequenced. They were identified as PKCs and annexin VI (see "Experimental Procedures").

A series of calmodulin mutants from Paramecium and chicken were tested for their ability to enhance Ca\(^{2+}\)-stimulated secretion, and none of the mutations abolished the calmodulin effect (data not shown). These mutations include S101F, M124Q (calmodulin N-terminal lobe mutants: E54K, G40E/V35I, D50N, and annexin VI (see "Experimental Procedures").
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Identification of Protein Kinase C as an Active Factor in the EGTA Extract—The material containing the high molecular mass (85 kDa) active factor after gel filtration chromatography was fractionated on a hydroxypatite column (Fig. 6). Careful comparison of the activity and SDS-PAGE profile of the fractions revealed that among all the visible silver-stained protein bands, only two have an elution profile similar to the activity (arrowheads in Fig. 6A). The fractions containing the activity (Fig. 6B) were pooled, concentrated, resolved on an 8% polyacrylamide gel and visualized by Coomassie staining (Fig. 6C). The two candidate bands were excised from the gel and sequenced. The 85-kDa band was identified as PKCa, and the 68-kDa band as annexin VI.

Since both PKC and annexins have been implicated in exocytosis (28, 48), we tested both for activity. Commercially purified rat brain PKC and recombinant human PKCa increased NE release, whereas commercially purified bovine liver annexin VI did not (Fig. 7). Western analysis using commercial PKC as standards indicated that PKC constitutes about 2% of total proteins in the EGTA extract and about 0.05% of total proteins in the cytosol (data not shown), demonstrating a 40-fold enrichment of PKC in the EGTA extract. A PKC inhibitory peptide (19–36) (IC\textsubscript{50} = 147 nm; used at 500 nM) inhibited 27% of EGTA extract triggered-release (6.7 μg of extract added).

The concentration of PKC needed for half-maximal increase in release was estimated to be about 3 nM (50 ng/200 μl). The effect of PKC is readily blocked by a competitive inhibitor of the ATP-binding site of PKC, bisindolylmaleimide I, and the PKC inhibitor peptide (19–36), which acts as a pseudo-substrate (data not shown), suggesting that PKC enhances NE release through ATP-dependent phosphorylation. Consistent with this, PKC increased release in the composite assay, but not in the triggering assay where ATP is absent (Fig. 8A). Analysis of PKC stimulation at different Ca\textsuperscript{2+} concentrations revealed a similar profile to calmodulin (Fig. 8B), suggesting that PKC's substrate protein is involved in the late, high [Ca\textsuperscript{2+}]\textsuperscript{1}\textsuperscript{requiring step of exocytosis. Therefore, PKC likely phosphorylates its substrate in the presence of ATP, and the substrate acts during the ATP-independent Ca\textsuperscript{2+}-triggering stage to increase NE release. Both PKC- and calmodulin-mediated effects on NE release utilize the conserved SNARE fusion machinery, as they could be completely abolished by addition of exogenous syntaxin H3 domains (data not shown). However, the same molecular pathway was not activated, since their effects were additive (data not shown).

DISCUSSION

In this study, we first identified an EGTA extract of brain membranes as a protein source capable of reconstituting Ca\textsuperscript{2+}-activated exocytosis in cracked PC12 cells. EGTA only extracts a small pool of Ca\textsuperscript{2+}-dependent membrane-associated proteins, and thus it served as an efficient initial purification step. Further protein chromatography led to the identification of two active factors in the starting extract, calmodulin and PKC, which together accounted for about half of the starting activity. Upon confirmation with commercially obtained proteins, this result unambiguously demonstrated that calmodulin and PKC mediate aspects of Ca\textsuperscript{2+}-dependent processes in exocytosis.

The finding that brain membrane EGTA extract alone is able to replace cytosol in supporting Ca\textsuperscript{2+}-triggered NE secretion in PC12 cells is somewhat surprising. We suggest that the likely explanation is 2-fold. First, some cytosolic proteins essential for exocytosis have a membrane-bound pool within permeabilized cells, whose activity might be sufficient for a normal level of
exocytosis. Second, although the 100,000 × g membrane pellet was washed to remove as many cytosolic proteins as possible, some cytosolic proteins that associate with membranes in a Ca\(^{2+}\)-independent manner are probably present in the membrane extract. However, these proteins likely constitute only a small percentage of the proteins in the extract, as the characteristics of the activity triggered by the membrane extract are quite different to that of cytosol (Fig. 2).

Using an unbiased biochemical purification method, we demonstrated that calmodulin and PKC directly modulate the exocytic machinery downstream of Ca\(^{2+}\) entry, and furthermore, that they signal through membrane-attached molecules to increase exocytosis. These targets include integral and peripheral membrane proteins, and cytosolic proteins that have a significant membrane-bound pool. The modest stimulation by calmodulin and PKC on secretion might suggest a regulatory role. However, it is also possible that some intermediates in their signaling pathways are in limiting amounts in the cell ghosts, so that their full effects were not observed. Half-maximal stimulation was obtained at about 3 nM for PKC and at about 75 nM for calmodulin. This is consistent with an enzymatic role for PKC, and predicts a high-affinity interaction between calmodulin and its substrate protein.

Ca\(^{2+}\) regulates exocytosis at many different levels. Prior studies indicated that Ca\(^{2+}\) signaling occurs in the priming steps as well as in triggering steps (49, 50). Our priming triggering protocol does not allow Ca\(^{2+}\)-dependent priming events to be assayed, as EGTA is present in the priming reaction. However, a different approach revealed the existence of both high and low Ca\(^{2+}\)-dependent processes (Fig. 2). Moreover, this analysis indicated that late triggering events require high [Ca\(^{2+}\)], whereas early priming events require low [Ca\(^{2+}\)]. If, as proposed, there is indeed a pronounced intracellular spatial and temporal [Ca\(^{2+}\)] gradient from the point of Ca\(^{2+}\) entry during depolarization (51), then, perhaps triggered events occur closer to the point of Ca\(^{2+}\) entry, while Ca\(^{2+}\)-dependent priming events occur further away from the point of Ca\(^{2+}\) entry. Distinct Ca\(^{2+}\) sensors at these stages might be appropriately tuned to different [Ca\(^{2+}\)] to handle different tasks. By analyzing the Ca\(^{2+}\) sensitivity of calmodulin- and PKC-stimulated release, we addressed the question of whether calmodulin and PKC plays an early or a late role in vesicle release. We showed that they both require relatively high [Ca\(^{2+}\)] (Fig. 5B), implying that calmodulin and PKC both mediate late triggering events, consistent with some earlier reports (34, 52, 53). In addition, it is interesting to note that PKC does not alter the calcium sensitivity of release in cracked cells, in contrast to observations from the chick ciliary ganglion (36). Therefore, in contrast to previous electrophysiological studies (28), we are able to limit the possible modes of PKC action in our system to an increase in the readily releasable vesicle pool or release sites, or an enhancement of the probability of release of individual vesicles upon Ca\(^{2+}\) influx.

The experiments assaying the calcium sensitivity of release (Figs. 2, 5, and 8) demonstrated a drop in release at very high [Ca\(^{2+}\)]. This decline in release at high [Ca\(^{2+}\)] has been previously reported (49, 51), and may represent the true Ca\(^{2+}\) sensitivity of the Ca\(^{2+}\)-sensing mechanism inside cells. However, in our system, it could also be due to the activation of a variety of Ca\(^{2+}\)-activated proteases, as experiments are usually performed in the presence of crude extracts, which include unsequenstered proteases.

What might the molecular targets of PKC and calmodulin be? An obvious calmodulin target molecule is CaMKII, as noted earlier. However, we showed that calmodulin’s effect on exocytosis is ATP-independent, rendering the involvement of a kinase extremely unlikely. Calmodulin has also been shown to associate with synaptic vesicles in a Ca\(^{2+}\)-dependent fashion through synaptotagmin (54), probably by binding to its C-terminal tail (55), and to promote Rab3A dissociation from synaptic vesicles (56). However, we found that there was little calcium-dependent binding of calmodulin to synaptotagmin either on synaptic vesicles, in a bead binding assay with recombinant proteins, or in a calmodulin overlay (data not shown). In addition, using immobilized calmodulin, we did not see significant Ca\(^{2+}\)-dependent pull-down of synaptotagmin or Rab3A from rat brain extract (data not shown). Recent work has suggested three other candidate targets for calmodulin, Munc13, Pollux, and CRAG (57). Pollux has similarity to a portion of a yeast Rab GTPase-activating protein, while CRAG is related to Rab3 GTPase exchange proteins. Further work is required to investigate the role of their interactions with calmodulin in vivo.

The recent report that calmodulin mediates yeast vacuole fusion (24) is intriguing, as it raises the possibility that calmodulin, a highly conserved ubiquitous molecule, may mediate many membrane trafficking events. It is not yet known if the effector molecule of calmodulin is conserved or variable across species and different trafficking steps. It is enticing to propose a model for Ca\(^{2+}\) sensing whereby calmodulin is a high affinity Ca\(^{2+}\) sensor for both constitutive and regulated membrane fusion. In the case of constitutive fusion, calmodulin may be the predominant Ca\(^{2+}\) sensor. In the case of slow, non-local exocytosis of large dense core granules, an additional requirement for the concerted actions of other molecule(s) that are better tuned to intermediate rises in [Ca\(^{2+}\)] might exist. At the highly localized sites of fast exocytosis of small clear vesicles where high [Ca\(^{2+}\)] is reached, specialized low affinity sensor(s) are likely required in addition to calmodulin to achieve membrane fusion. Therefore, although calmodulin participates in multiple types of vesicle fusion, the impact of Ca\(^{2+}\) sensing by calmodulin on vesicle release likely varies.

Due to the fact that calmodulin binding to some proteins can be modulated by PKC phosphorylation, one might suspect that PKC action on exocytosis proceeds through a calmodulin-dependent pathway. However, we found that the effects of calmodulin and PKC are additive within our system, suggesting that PKC does not act by releasing calmodulin from a substrate that functions as a calmodulin storage protein. How Ca\(^{2+}\) regulates presynaptic vesicle release has been an open question for many years. By identifying calmodulin and PKC as modulators of Ca\(^{2+}\)-regulated exocytosis and clarifying their functions, we have extended our knowledge of the release process. While the basic machinery of membrane fusion is becoming better understood, the multiple effects of Ca\(^{2+}\) on exocytosis remain to be elucidated at the molecular level. In addition, the ways that Ca\(^{2+}\) regulation may be important to the mechanisms of synaptic plasticity in the central nervous system will be a major focus in the future.

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