Resolution of Regulated Secretion into Sequential MgATP-dependent and Calcium-dependent Stages Mediated by Distinct Cytosolic Proteins

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Abstract. The biochemical events and components responsible for ATP-dependent Ca^{2+}-activated secretion remain to be identified. To simplify the molecular dissection of regulated secretion, we have resolved norepinephrine (NE) secretion from semi-intact PC12 cells into two kinetically distinct stages, each of which was studied separately to discern its molecular requirements. The first stage consisted of MgATP-dependent priming of the secretory apparatus in the absence of Ca^{2+}. MgATP-dependent priming was readily reversible and inhibited by a broad range of protein kinase inhibitors. The second stage consisted of Ca^{2+}-triggered exocytosis which, in contrast to priming, occurred in the absence of MgATP. Both priming and triggering were found to be dependent upon or stimulated by cytosolic proteins. The priming and triggering activities of cytosol were functionally distinct as indicated by differing thermolability. Furthermore, active components in cytosol resolved by gel filtration were found to support either priming or triggering, but not both. For both priming and triggering reactions, several peaks of activity were detected; one of each type of factor was partially purified from rat brain cytosol, and found to be enriched for stage-specific activity. Two partially purified factors exhibiting stage-specific activity, a ~20-kD priming factor and ~300-kD triggering factor, were able to support regulated secretion as effectively as crude cytosol when used sequentially in the partial reactions. Further characterization of stage-specific cytosolic factors should clarify the nature of MgATP- and Ca^{2+}-dependent events in the regulated secretory pathway.

Two major obstacles to biochemical studies of regulated secretion are the inaccessibility of the secretory apparatus in intact cells, and the failure of cell-free systems to preserve the structural integrity required for secretory function. These obstacles have been overcome with permeabilized cell models, which have revealed some general biochemical requirements for regulated secretion. Norepinephrine (NE)[1] secretion from digitonin-permeabilized adrenal chromaffin cells has been found to be dependent on Ca^{2+} and MgATP (Dunn and Holz, 1983; Wilson and Kirshner, 1983) as well as cytoplasmic proteins (Sarafian et al., 1987). Recent studies have begun to identify and characterize the cytoplasmic proteins required for regulated secretion from permeabilized cells (Ali et al., 1989; Martin and Walent, 1989; Wu and Wagner, 1991; Sarafian et al., 1991; Morgan and Burgoyne, 1992; Walent et al., 1992).

The biochemical events responsible for ATP-dependent Ca^{2+}-activated secretion remain to be elucidated. However, some progress has been made in ordering the ATP- and Ca^{2+}-dependent steps relative to one another. Holz and collaborators suggested that MgATP may act before Ca^{2+} to maintain a "primed" state of the secretory apparatus in digitonin-permeabilized chromaffin cells (Holz et al., 1989). In streptolysin-*O*-permeabilized mast cells, Howell et al. (1989) found that ATP was required to maintain a high affinity of the response system for Ca^{2+} and GTPγS, but was not required for the final stages of triggered exocytosis. Similarly, in *Paramecium* it was concluded that ATP was required to prime the system but was not required for the exocytotic event per se (Vilmart-Seuwen et al., 1986; Lumpert et al., 1990). These studies in diverse systems suggest that ATP acts before Ca^{2+} in the pathway of regulated secretion. However, a requirement for cytoplasmic proteins in potentially distinct ATP- and Ca^{2+}-dependent steps has not been investigated.

Other membrane trafficking events have been successfully reconstituted and dissected in vitro. Cell homogenates and semi-intact cells have been used to study vesicle-mediated protein transport from the ER to Golgi (Beckers et al., 1987; Baker et al., 1988; Ruohola et al., 1988), intra-Golgi transport (Balch et al., 1984), endosome fusion (Braell, 1987), and fusion of constitutive vesicles with the plasma membrane (Helms et al., 1990; Miller and Moore, 1991). In each of these cases, a requirement for cytoplasmic proteins has

1. *Abbreviation used in this paper*: NE, norepinephrine.

been established, and several of these proteins have been further characterized (reviewed by Rothman and Orci, 1992). Additionally, membrane transport processes have been further dissected into the sequential formation and consumption of several intermediates within an overall transport step. These putative intermediates have been detected based either on their accumulation at reversible transport blockpoints or their passage beyond points of sensitivity to inhibitors or requirements for specific proteins. In intra-Golgi transport, an intermediate was identified whose consumption required much less cytosol than its production (Wattenberg et al., 1986). Cytosolic factors operative in the production and consumption of the intermediate were resolved by chromatography (Wattenberg and Rothman, 1986).

In the present study, we describe the resolution of MgATP-dependent Ca^{2+}-activated secretion into two sequential stages based on different requirements for Ca^{2+} and MgATP. During the first stage (priming), a labile MgATP-dependent intermediate accumulates in the absence of Ca^{2+}. Ca^{2+}-dependent consumption of this intermediate (triggering) results in rapid MgATP-independent secretion. Semi-intact NE-secreting PC12 cells were used for these studies. Because the semi-intact cells can be easily depleted and reconstituted with cytosolic proteins, it was possible to demonstrate that both priming and triggering were dependent upon or stimulated by cytosolic proteins. Furthermore, it was shown that the active cytosolic proteins supported either priming or triggering, but not both. Several peaks of stage-specific activity were resolved by gel filtration, and two were further purified, a ~20-kD priming factor and a ~300-kD triggering factor.

Materials and Methods

Preparation of Semi-intact PC12 Cells

PC12 cells were maintained on 100-mm dishes in DME essentially as described (Lomneth et al., 1991). Cells were subcultured every 5 d and used in experiments between passages 25 and 60.

Cells were labeled with [^{3}H]NE by supplementing the growth medium with 0.5 μCi/ml [^{3}H]NE (Amersham Corp., Arlington Heights, IL) and 0.5 mM sodium ascorbate for an overnight incubation at 37°C (12–16 h). Unincorporated [^{3}H]NE was removed by aspirating the medium and rinsing the cells twice with 8 ml of DME containing 0.1% BSA in place of serum. The cells were further rinsed twice by incubation for 1 h with the BSA-containing DME at 37°C.

Cells were depleted of intracellular ATP by rinsing them twice with PC12 buffer (25 mM Hepes, pH 7.3, 70 mM sucrose, 130 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, and 1.2 mM $MgSO_4$) and incubating for 10 min at 37°C with PC12 buffer containing 0.2 mM iodoacetic acid (sodium salt) and 0.1 μM antimycin A. This ATP depletion step reduced the MgATP-independent secretion from semi-intact cells and thus accentuated their dependence on MgATP-dependent priming. However, this treatment did not affect the optimal secretory output of the semi-intact cells, nor was it required for priming to be observed. For example, the experiment in Fig. 4 used semi-intact cells prepared from PC12 cells not treated with metabolic inhibitors.

Cells were harvested from the dishes by pipetting with ice-cold KGlu buffer (20 mM Hepes, pH 7.2, 120 mM potassium glutamate, 20 mM potassium acetate, 2 mM EGTA, and 0.1% BSA). Subsequent manipulations were at 0–4°C unless noted. The cells were permeabilized by a single passage through a stainless steel ball homogenizer consisting of a 0.3747-inch bored chamber and a 0.3746-inch tungsten carbide ball. This procedure, called "cell cracking," results in a large tear in the surface membrane of cells (Martin, 1989; Martin and Walent, 1989). Despite a high degree of permeability to solutes and macromolecules, cracked cells retain intracellular functional and structural integrity (Martin, 1989; Martin and Walent, 1989; Lomneth et al., 1991). Permeabilization was 95–100% as assessed by trypan blue staining. After permeabilization, semi-intact cells were adjusted to 11 mM EGTA and incubated 1–2 h to fully extract soluble components. Semi-intact cells were then rinsed 3 times by centrifugation at 800 g for 5 min and resuspension in KGlu buffer.

Priming and Triggering Incubations

Priming incubations were conducted for 30 min at 30°C in 200 μl KGlu buffer containing 2×10^6 semi-intact cells, 2 mM MgATP, and ~1 mg/ml rat brain cytosol. When larger numbers of primed semi-intact cells were needed, priming reactions were scaled up proportionally. Priming was found to be optimal by 30 min of incubation (not shown). The EC_{50} of priming for MgATP was ~300 μM (not shown). Batches of rat brain cytosol varied in their specific activities, so optimal doses were determined for each batch. For most experiments crude rat brain cytosol was used, but in others we substituted dialyzed rat brain cytosol (cutoff: 12–14 kD), bovine adrenal medulla cytosol, or partially purified cytosolic proteins.

Priming reactions were terminated by chilling 3 min on ice, and semi-intact cells were rinsed free of MgATP and cytosol by centrifugation for 1 min at 2,000 g at 4°C and resuspension in KGlu buffer. A single centrifugation and resuspension reduced MgATP levels sufficiently to prevent residual MgATP from contributing to triggering reactions (see Fig. 3). However, in many experiments, one or two additional washes were used to remove residual cytosolic proteins.

Triggering was conducted for 3 min at 30°C in 200 μl of KGlu buffer containing 1×10^6 semi-intact cells, 1 μM free ionic Ca^{2+} (calculated as in Ronning and Martin, 1985), and 0.5–1.0 mg/ml rat brain cytosol. The EC_{50} of triggering for Ca^{2+} was ~200 nM (not shown). The optimal dose of cytosol varied between cytosol batches. Triggering was carried out with crude cytosol, dialyzed cytosol, or partially purified cytosol proteins.

Triggering incubations were terminated by chilling 3 min on ice, followed by centrifugation at 2,000 g for 40 min at 4°C. The released [^{3}H]NE in the supernatant was removed and quantitated by scintillation counting. Cell pellets were dissolved in 1% Triton X-100 to quantitate the [^{3}H]NE retained by the cells. A typical triggering reaction had a total of $5–10 \times 10^3$ cpm (^{3}H) in the supernatant and pellet. 98% of the radioactivity retained and released by the semi-intact cells was present as NE (Lomneth et al., 1991).

Preparation of Cytosol

Cytosol was prepared by homogenization of tissue in ~3 vols of ice-cold homogenization buffer consisting of 20 mM Hepes, pH 7.0, 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA, 0.1 mM DTT, 0.4 mM PMSF, and 0.5 μg/ml leupeptin. Homogenates were centrifuged at 30,000 g for 30 min followed by centrifugation at 100,000 g for 90 min, at 4°C. Cytosols were stored at −20°C.

Gel Filtration Chromatography

Cytosols were concentrated to 30–40 mg/ml and 2 ml was loaded onto a 50 cm × 2 cm^2 Superose 12 column (Pharmacia LKB, Uppsala, Sweden) equilibrated with KGlu buffer lacking BSA at a flow rate of 0.5 ml/min. 2-min fractions were collected starting 60 min after injection. Fractions were held on ice for 1 wk or more while experiments were conducted, with no apparent loss of activity. The overall recoveries of priming and triggering activity in the gel filtration experiments shown in Fig. 7 were 21 and 128%, respectively, for adrenal, and 80 and 2%, respectively, for brain. These values should be considered approximate since the yield of activity appeared to vary significantly depending upon the volume of each fraction assayed, indicating that the assay conditions were nonlinear. The values listed were calculated using the most limiting volumes tested, however, equally limiting volumes were not tested in each case. Also, the degree to which inhibitory cytosolic components affected the apparent recovery of activity is not known.

Purification of Cytosolic Priming and Triggering Factors

The partial purification of a 20-kD priming factor was carried out in conjunction with a p145 purification (Walent et al., 1982), using the same starting material and initial chromatography steps. Cytosol from 500 rat brains was prepared as above, except that the following additional protease inhibi-

tors were included in the homogenization buffer: 2.5 μg/ml aprotinin, 0.5 μg/ml E-64, and 1 μg/ml calpain inhibitor II (Boehringer Mannheim GmbH, Mannheim, Germany). The cytosol (~1.35 liters) was diluted to 2 liters with deionized water, loaded onto a 500-ml DEAE cellulose column (resin DE52, Whatman Ltd., Maidstone, England) equilibrated with DEAE buffer (20 mM Tris, pH 7.0, 2 mM EGTA, 0.1 mM DTT, 0.4 mM PMSF, and 0.5 μg/ml leupeptin), and the flowthrough was collected. Bound proteins were then eluted with DEAE buffer containing 0.3 M KCl. Both DEAE flowthrough and eluted pools contained priming activity, indicative of the presence of several distinct priming factors in brain cytosol. Only the 0.3 M KCl eluate was carried forward. After extensive dialysis against DEAE buffer, the fraction was adjusted to 6 mM $CaCl_2$ and 20 μg/ml leupeptin, loaded onto a 400-ml phenyl-Sepharose column equilibrated in A buffer (20 mM Tris, pH 7.0, 2 mM $CaCl_2$, 0.1 mM DTT, 0.4 mM PMSF, and 0.5 μg/ml leupeptin), and the flowthrough was collected. The column was eluted sequentially with B buffer (A buffer plus 1 M NaCl), and C buffer (A buffer lacking $CaCl_2$ and containing 2 mM EGTA). Most of the recovered priming activity was in the C buffer eluate. Further washing of the column with E buffer (A buffer plus 50% ethylene glycol) and D buffer (C buffer plus 50% ethylene glycol) failed to elute significant priming activity. At this point, the purification of priming activity differed from the p145 purification which used the D wash from phenyl-Sepharose (Walent et al., 1992). Priming activity in the C fraction was concentrated and gel filtered on Superose 12 as described above. >83% of the recovered priming activity eluted with an apparent molecular mass of 20 kD. Fractions from the 20-kD region were analyzed by SDS-PAGE, and found to be simplified relative to cytosol, consisting of at least 10 protein bands (not shown). Only one protein band (32 kD) identically co-eluted with the priming activity; however, it remains to be established whether that protein is responsible for activity. A pool of the active fractions was characterized for priming and triggering activity (see Fig. 9 *A*).

p145-containing fractions generated by DEAE cellulose and Ca^{2+}-dependent phenyl-Sepharose chromatography (see above) were further purified by Matrex Green dye affinity chromatography (Amicon Div. W. R. Grace and Co., Danvers, MA) as described (Walent et al., 1992). Fractions at this level of purity (5–15%) from several p145 purifications were tested for priming and triggering activity. See Fig. 9 *B* for results from a representative experiment.

Data Presentation

Basal NE release and cell losses during priming and associated manipulations affected the total [^{3}H]NE present during triggering. To compensate for differences in the total cell-associated [^{3}H]NE added to triggering reactions, the secreted [^{3}H]NE was expressed as a percent of the total [^{3}H]NE in each individual reaction. The observed effects of priming and triggering were not dependent upon data normalization. When the data were expressed as [^{3}H]NE secreted on a per cell basis, the results were qualitatively similar.

For experiments involving cytosolic protein stimulation of NE release, the results are expressed as the percent of maximal cytosol-dependent activity: the difference in percent [^{3}H]NE released between omission and inclusion of a particular fraction was divided by the difference between omission and inclusion of a maximally effective quantity of rat brain cytosol, and multiplied by 100. For recovery estimates, 1 U of priming or triggering activity was defined as the activity required to stimulate NE secretion by 1% of the maximal cytosol-dependent activity.

Experiments shown are representative of at least two similar experiments. Most of the data represent the mean of duplicate determinations within the same experiment. The range is indicated by a bar when larger than the symbol size.

Materials

Unless otherwise noted, chemical reagents were from Sigma Chemical Co. (St. Louis, MO). Okadaic acid and staurosporine were from Calbiochem Corp. (LaJolla, CA), lavendustin A and genistein were from Gibco BRL (Gaithersburg, MD), and K252a was from Kamiya (Thousand Oaks, CA).

H-7 was purchased from Sigma Chemical Co. After completion of this work, Sigma Chemical Co. reported that H-7 produced in their laboratories appeared to be 1-(5-isoquinolinylsulfonyl)-3-methylpiperazine rather than 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine as originally described (Hidaka et al., 1984).

Results

Sequential MgATP- and Ca^{2+}-dependent Steps in Regulated Secretion from Semi-intact PC12 Cells

Permeabilization by cell cracking results in the virtually complete depletion of soluble cytoplasmic factors with maintenance of a high degree of normal cytoplasmic ultrastructure (Martin, 1989; Martin and Walent, 1989). Secretion from washed semi-intact GH3 and PC12 cells permeabilized by this method was found to require Ca^{2+}, MgATP, and cytosol proteins (Martin and Walent, 1989; Lomneth et al., 1991). However, Ca^{2+}-activated secretion from semi-intact PC12 cells was not completely dependent on MgATP addition. About half of the optimal Ca^{2+}-activated [^{3}H]NE secretion elicited by Ca^{2+}, MgATP, and cytosol occurred in the absence of added MgATP (Fig. 1 *B*, ○ vs Fig. 1 *A*, △). A possible explanation for this MgATP-independent component was that MgATP and Ca^{2+} acted separately in sequential steps, and that MgATP-independent secretion arose from an intermediate which had already passed the MgATP-dependent step(s). A similar explanation was originally suggested by Holz et al. to account for a MgATP-independent component of Ca^{2+}-activated secretion from digitonin-permeabilized chromaffin cells (Holz et al., 1989).

If MgATP acted before Ca^{2+}, it should be possible to reconstitute the MgATP-dependent and Ca^{2+}-dependent steps sequentially. To test whether secretion could pass the MgATP-dependent step(s) in the absence of Ca^{2+}, semi-intact PC12 cells were preincubated with MgATP and cytosol in an EGTA buffer containing ≤1 nM-free Ca^{2+}. We refer to the MgATP-preincubated semi-intact cells as "primed." As shown in Fig. 1, Ca^{2+}-activated NE secretion from primed (▲●■) and unprimed (△○□) semi-intact cells was markedly different. Priming with MgATP enhanced the initial rate of optimal Ca^{2+}-activated (Fig. 1 *A*, ▲ vs △) NE secretion. Significantly, both the initial rate and final extent of MgATP-independent Ca^{2+}-activated (Fig. 1 *B*, ● vs ○) NE secretion were greatly enhanced by priming. The increase in the initial rate and MgATP-independence of Ca^{2+}-activated NE secretion indicated that a rate-limiting MgATP-requiring step occurred during the priming preincubation with MgATP and cytosol.

Enhanced Ca^{2+}-activated secretion from primed semi-intact cells may represent the consumption of a MgATP-dependent intermediate generated during the preincubation. Since the enhanced secretion was very rapid and MgATP independent, optimal detection of this component used brief MgATP-free "triggering" incubations containing Ca^{2+} and cytosol. Priming and triggering incubations are shown schematically in Fig. 2.

Priming and triggering incubations resolved MgATP-dependent Ca^{2+}-activated secretion into two sequential stages, either of which could be independently manipulated. As shown in Fig. 3, both stages were required for the reconstitution of Ca^{2+}-activated NE release since leaving the reaction at a reduced temperature during either stage resulted in a marked decrease in NE secretion (bars *1* vs *5* and *2* vs *5*). Priming was entirely dependent upon MgATP (bar *3* vs *5*), whereas triggering only occurred in the presence of Ca^{2+} (bar *4* vs *5*). Only MgATP served as a substrate for priming; MgGTP, MgCTP, and MgITP failed to substitute (Table I). Both mag-

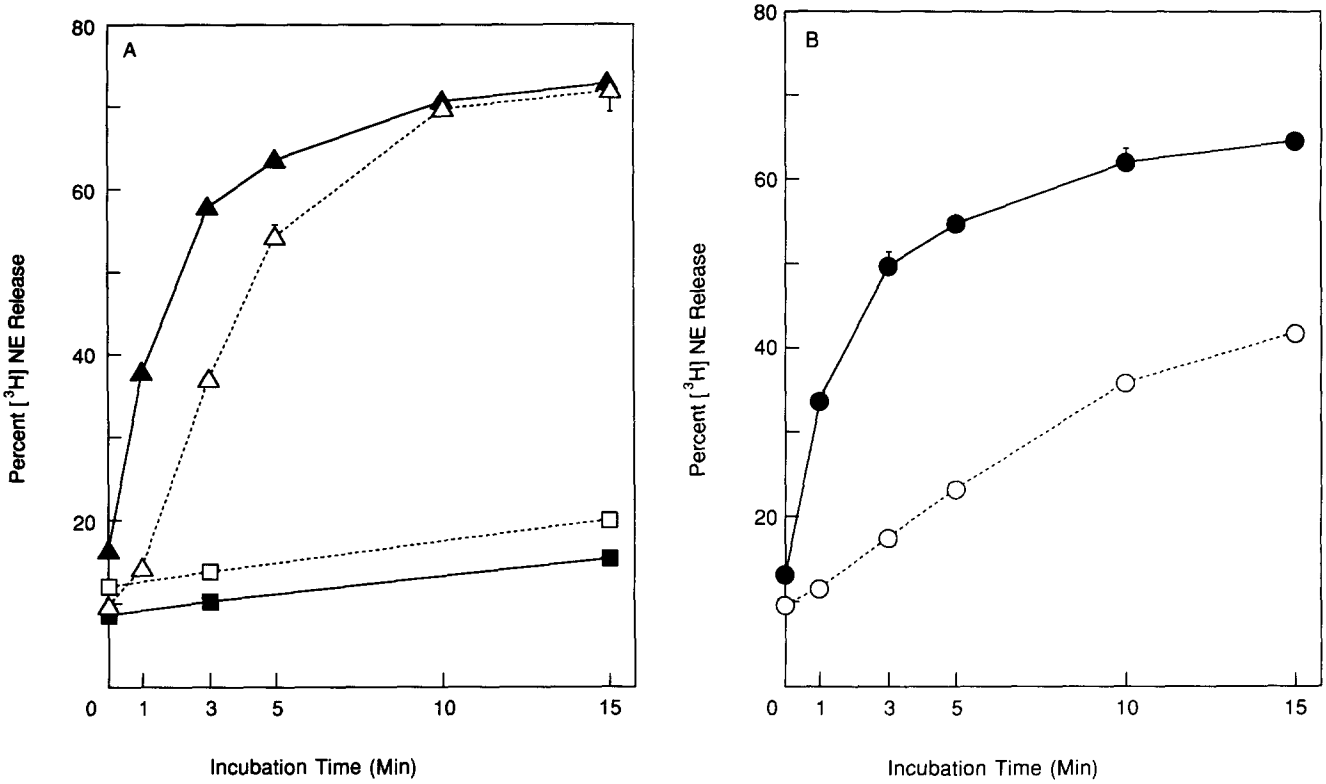

Figure 1. Time course of Ca^{2+}-activated NE secretion from MgATP-primed and unprimed semi-intact cells. Semi-intact cells were either primed (▲●■) by preincubation with 2 mM MgATP and 1 mg/ml rat brain cytosol, or left on ice as a control (△○□). Primed semi-intact cells were centrifuged and resuspended to remove MgATP and cytosol. Aliquots of 1×10^6 primed or unprimed semi-intact cells were distributed to secretion reactions of 3 types: (*A*) optimal Ca^{2+}-activated (▲△) secretion incubations containing 1 μM free ionic Ca^{2+}, 2 mM MgATP, and 0.5 mg/ml rat brain cytosol; basal (■□) incubations with KGlu buffer only; and (*B*) MgATP-independent Ca^{2+}-activated (●○) incubations containing Ca^{2+} and cytosol. Secretion reactions were incubated for the indicated times at 30°C, chilled, and [^{3}H]NE release was determined.

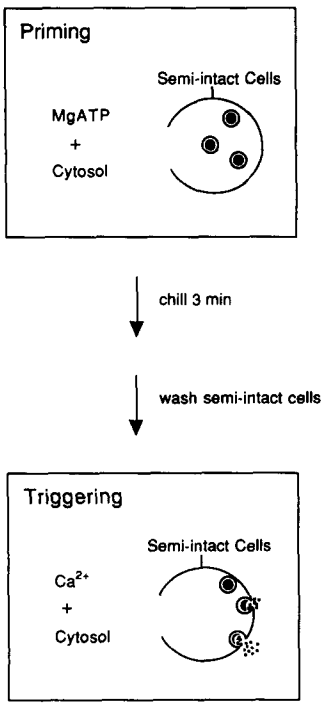

Figure 2. Diagram of the sequential partial reactions representing regulated secretion. MgATP is shown to prime semi-intact cells for the process of Ca^{2+}-triggered secretion. Both priming and triggering require the presence of cytosolic proteins. Priming, 30 min, 30°C, triggering, 3 min, 30°C.

nesium and ATP were required for MgATP-dependent priming, and nucleotide hydrolysis appeared to be required since the nonhydrolyzeable ATP analogs adenylylimidodiphosphate and adenylyl (β,γ-methylene) diphosphonate did not support priming (not shown).

An important aspect of this two-stage system was that the essential components for either partial reaction were excluded from the other. Ca^{2+}-dependent processes were excluded during priming by the presence of 2 mM EGTA, and MgATP-dependent processes were excluded during triggering by the prior wash-out of nucleotides. The lack of dependence of triggering on MgATP did not result from residual MgATP from priming preincubations or from cytosol, nor from ATP production by the semi-intact cells, since inclusion of a MgATP depletion system during triggering failed to diminish secretion from primed semi-intact cells (Fig. 3, bar *5* vs *7*). More extensive washing of primed semi-intact cells or the use of dialyzed cytosol during triggering reactions did not diminish secretion, further indicating that MgATP-dependent events did not contribute to triggering (not shown). A previous study had determined that the available MgATP levels in washed semi-intact PC12 cells were ≤ 10 μM, well below the concentration required to stimulate secretion (Lomneth et al., 1991).

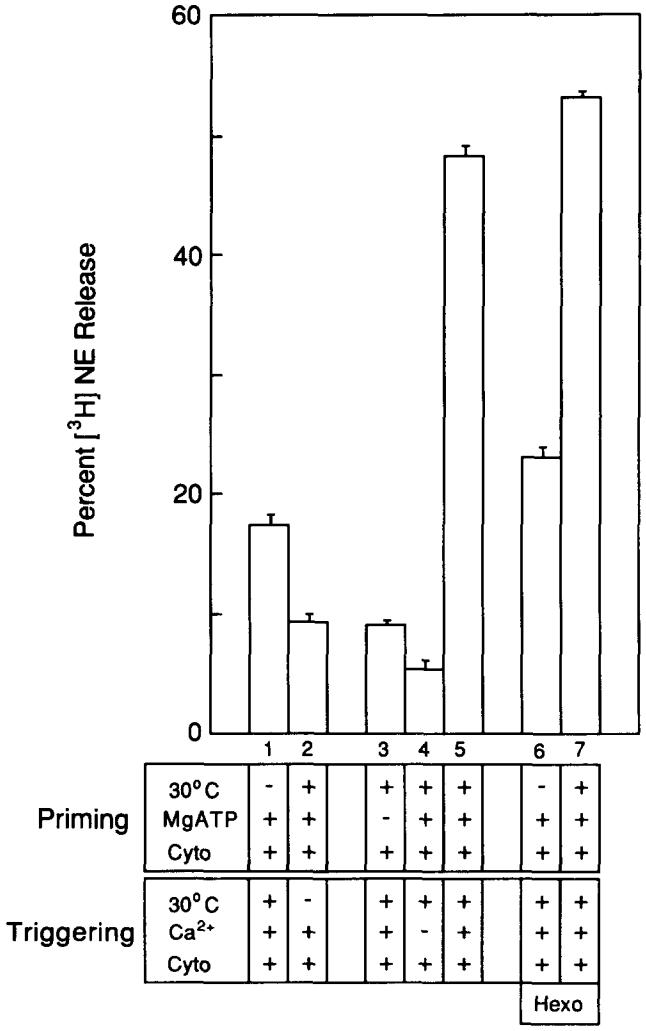

Figure 3. Dependence of priming and triggering on elevated temperature, MgATP, and Ca^{2+}. To enable the NE secretion shown in bar *5*, all requirements for priming and triggering were provided. Bars *1–4* show the result of omitting one of the required components from either priming or triggering reactions: bar *1*, priming reaction was held on ice; bar *2*, triggering reaction was held on ice; bar *3*, MgATP was omitted from priming reaction; bar *4*, Ca^{2+} was omitted from triggering reaction. Bars *6* and *7* demonstrate that triggering was not dependent upon possible trace amounts of MgATP present in the absence of MgATP addition. After priming (bar *6*, priming reaction was held on ice; bar *7*, priming was conducted normally), semi-intact cells were centrifuged, resuspended, and added to triggering reactions supplemented with 5 U/ml hexokinase, 10 mM glucose, and 2 mM $MgCl_2$. The hexokinase-containing triggering reactions were incubated at 30°C for 4 min: 1 min in the absence of Ca^{2+}, followed by the usual 3 min after Ca^{2+} addition. The 1 min before Ca^{2+} addition was included to allow hexokinase time to hydrolyze trace MgATP before secretion was triggered. Hexokinase was capable of depleting millimolar quantities of MgATP under these conditions, as assessed by nucleotide analysis with TLC (not shown). Inclusion of hexokinase activity resulted in a slight enhancement of Ca^{2+}-triggered NE release.

Priming Is Readily Reversible

The MgATP-dependent intermediate formed during priming was found to be unstable. When primed semi-intact cells were incubated without MgATP, their enhanced capacity for rapid MgATP-independent Ca^{2+}-activated secretion was readily lost. This reversal of priming was termed "depriming." In the experiment shown in Fig. 4 *A*, semi-intact cells were preincubated for 30 min under priming (•) or depriming (○) conditions. After 30 min, both sets of cells were shifted to the other condition, and the preincubations were continued for another 30 min. At various times during the preincubations, semi-intact cells were tested in triggering reactions. The priming and depriming incubations were found to enhance and diminish, respectively, the rapid secretion triggered by Ca^{2+} and cytosol. These results indicated that MgATP-dependent priming was readily reversible.

It was important to further establish that depriming specifically reversed the effects of priming rather than generally reduced the secretory capacity of semi-intact cells. Since priming had increased the MgATP-independence of secretion without affecting the final extent of optimal secretion (Fig. 1), depriming should also affect only the MgATP independence. Semi-intact cells from the experiment in Fig. 4 *A* were tested in full-length 15-min secretion incubations to determine their capacity for secretion with and without MgATP. In contrast to triggering reactions, the long incubations allowed secretion to proceed to its final extent. As shown in Fig. 4 *B*, optimal Ca^{2+}-activated (▲,△) secretion elicited by Ca^{2+}, MgATP, and cytosol was not markedly altered by priming and depriming incubations. In contrast, all of the MgATP-independent secretion was subject to regulation by the priming/depriming mechanism (Fig. 4 *C*, •,○). These results further indicated that depriming represents the reversal of MgATP-dependent priming.

Table I. Nucleotide Specificity and Protein Kinase Inhibitor Sensitivity of Priming

	Priming
	Percent of control
Nucleotides*	
1 mM MgATP	100
1 mM MgGTP	7 ± 4
1 mM MgITP	0 ± 4
1 mM MgCTP	−4 ± 4
Protein kinase inhibitors‡	
none	100
1 μM lavendustin A	90 ± 7
100 μM genistein	26 ± 5
100 μM H-7§	38 ± 3
0.3 μM staurosporine	30 ± 3
0.5 μM K252a	87 ± 8
1.0 mM 6′-DMAP‖	−11 ± 2
1.0 mM FSBA	−4 ± 6

* Priming incubations were carried out with rat brain cytosol and the indicated nucleotides. After priming, semi-intact cells were centrifuged and resuspended to remove the nucleotides, and Ca^{2+}-activated secretion was determined with triggering reactions. The difference in Ca^{2+}-triggered secretion from semi-intact cells primed in the absence of nucleotides (12% NE release) and the presence of 1 mM MgATP (42% NE release) was defined as 100%.
‡ Priming incubations containing rat brain cytosol and 1 mM MgATP were carried out in the presence or absence of the indicated inhibitors. After priming, semi-intact cells were centrifuged and resuspended twice to remove cytosol, MgATP, and inhibitors, and Ca^{2+}-activated secretion was determined with triggering reactions. The difference in Ca^{2+}-triggered secretion obtained with unprimed control semi-intact cells (14% NE release) and primed semi-intact cells not exposed to inhibitors (37% NE release) was defined as 100%.
§ See note on the structure of H-7 in Materials and Methods.
‖ Negative priming values may represent the prevalence of depriming when MgATP-dependent priming is effectively blocked.

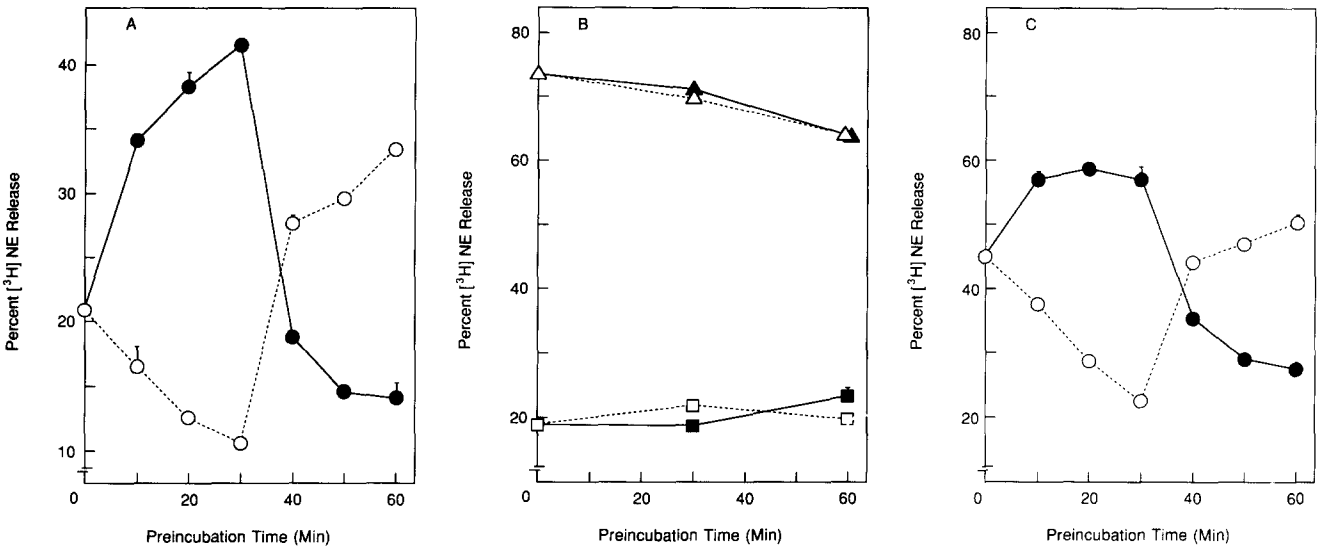

Figure 4. Priming is readily reversible. Semi-intact cells were prepared as usual except that the incubation with metabolic inhibitors before permeabilization (see Materials and Methods) was omitted. Semi-intact cells were split into 2 groups: one group was preincubated under priming (▲●■) conditions, the other under depriming (△○□) conditions. Depriming conditions were identical to those for priming, but lacked added MgATP. After 30 min, both groups of cells were chilled, centrifuged, resuspended, and switched to the other condition, and the preincubations were continued for another 30 min. At 10-min intervals during the preincubations, a portion of the semi-intact cells were centrifuged, resuspended, and distributed to 3-min triggering incubations (*A*) or full-length 15-min secretion incubations (*B* and *C*), and NE secretion was determined. The 15-min secretion incubations used optimal Ca^{2+}-activated (+ Ca^{2+} + MgATP + Cyto) (▲△), MgATP-independent Ca^{2+}-activated (+ Ca^{2+} − MgATP + Cyto) (●○), or basal (− Ca^{2+} − MgATP − Cyto) (■□) secretion conditions, as defined in the legend to Fig. 1. The dramatic effect of depriming on NE secretion was found to be at least partially dependent upon cytosol addition to the preincubations (not shown).

Protein Kinase Inhibitors Block Priming

The characteristics of the priming reaction were consistent with a reversible ATP-dependent modification such as protein phosphorylation. To determine whether priming and depriming represent protein phosphorylation and dephosphorylation, respectively, several general protein kinase and protein phosphatase inhibitors were tested. As shown in Table I, the following protein kinase inhibitors significantly inhibited priming: H-7 (Hidaka et al., 1984), staurosporine (Tamaoki et al., 1986; Nakano et al., 1987), genistein (Akiyama et al., 1987), 6′-dimethylaminopurine (6′-DMAP) (Neant and Guerrier, 1988), and 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA) (Colman, 1983). The results with kinase inhibitors are consistent with protein phosphorylation being required for MgATP-dependent priming. However, the inhibitory drugs interact with the ATP-binding site of protein kinases, hence the possibility that other ATP-binding proteins are inhibited seems at least equally likely. Additionally, the protein phosphatase inhibitors 1.2 μM okadaic acid (Biolojan and Takai, 1988; Haystead, et al., 1989), 25 mM potassium fluoride (Brautigan and Shriner, 1988), and 1.0 mM orthovanadate (Swarup et al., 1982) failed to influence the depriming of primed semi-intact cells (not shown). These results render unlikely the involvement of numerous protein phosphatases in depriming.

Priming and Triggering Require Distinct Cytosolic Proteins

Both priming and triggering reactions were found to be dependent upon or stimulated by cytosolic factors (Fig. 5). Priming was strongly cytosol dependent since addition of cytosol to priming preincubations stimulated Ca^{2+}-activated secretion during the triggering incubation by >100% (Fig. 5 *A*, bar *3* vs *5*). By comparison, the triggering reaction appeared to be less cytosol dependent (bar *4* vs *5*). However, the triggering activity of cytosol required a primed intermediate since cytosol addition to triggering reactions using unprimed semi-intact cells failed to support Ca^{2+}-activated NE release (bar *1* vs *2*).

As shown in Fig. 5 *B*, the triggering reaction required less cytosol than the priming reaction, indicating that priming factors were relatively more limiting in cytosol. In the experiment shown, half-maximal triggering was observed with ~10 μg of cytosol protein, whereas half-maximal priming required ~35 μg. Previous findings indicated that the cytosolic factors required for regulated secretion were proteins (Martin and Walent, 1989). Cytosol priming and triggering activities were found to be thermolabile (see below) and were not affected by dialysis (not shown), consistent with a protein nature.

Since priming and triggering reactions distinctively required MgATP or Ca^{2+}, respectively, but not both, it seemed plausible that different cytosolic activities, and perhaps distinct proteins, were operative in these stages. The thermolability of cytosol priming and triggering activities was examined to test this suggestion. Incubations at above 49°C eliminated the priming and triggering activities of cytosol, indicating that priming and triggering were mediated by thermolabile proteins (not shown). However, incubations at 40–49°C caused a greater loss of priming activity than triggering activity. As shown in Fig. 6, the priming activity of cytosol was completely abolished by a 46°C pretreatment, whereas triggering activity was unaffected. These results demonstrated

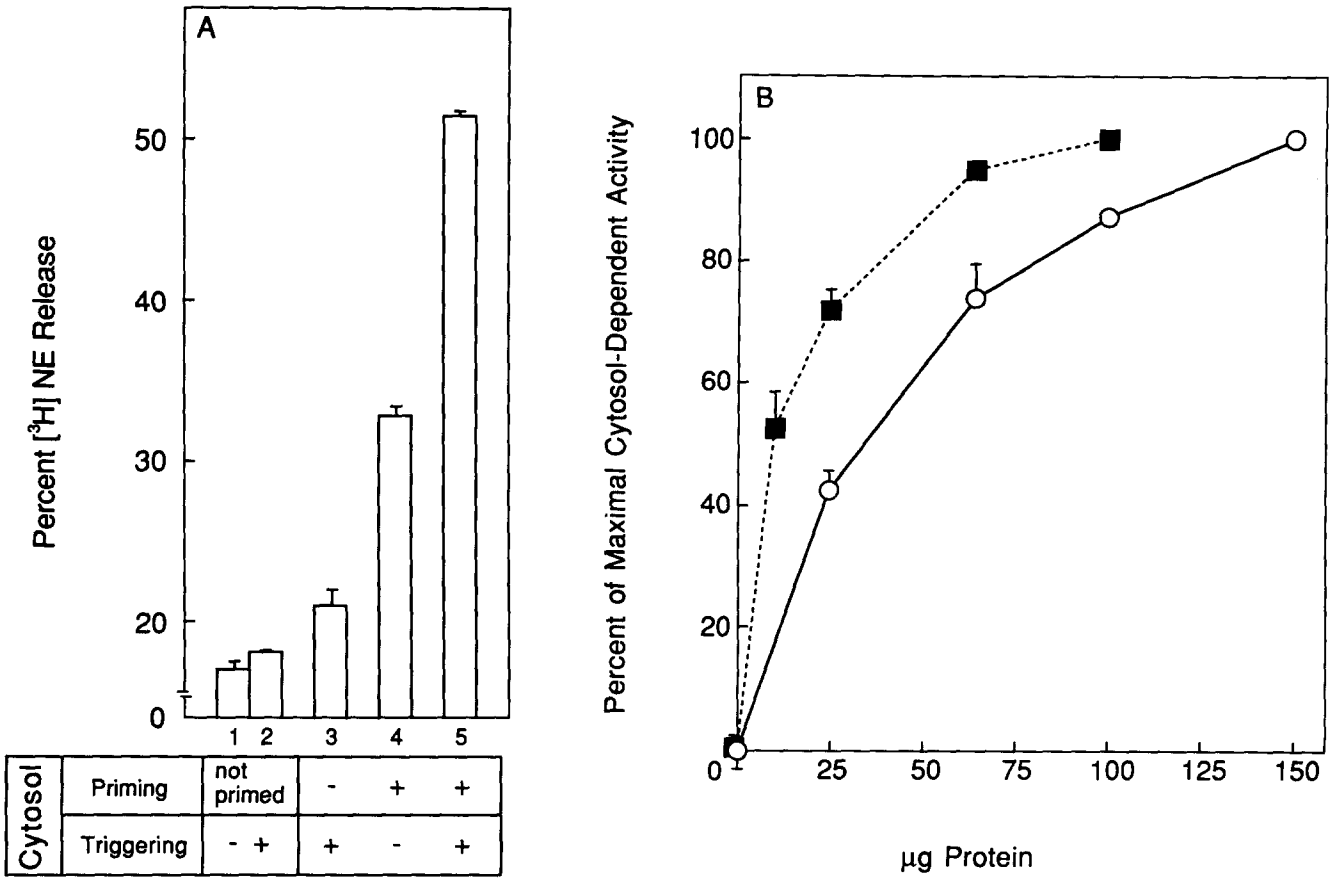

Figure 5. Cytosol dependence of priming and triggering. Using an optimally effective amount of rat brain cytosol in one partial reaction, the effect of cytosol addition to the complementing partial reaction was determined. *A* shows the effect on NE secretion of omitting (−) or adding (+) a saturating amount of rat brain cytosol to priming (bar *3* vs *5*) or triggering (bar *4* vs *5*) incubations. Also shown is the effect of cytosol addition to triggering incubations using unprimed semi-intact cells (bar *1* vs *2*). *B* shows cytosol stimulation of NE secretion as a function of protein amount in priming (○) or triggering (■) reactions. Data expressed in *A* and *B* are from the same experiment.

that priming and triggering were mediated by distinct thermolabile protein activities.

To determine the complexity and approximate molecular weights of priming and triggering factors, gel filtration of crude cytosol from two secretory tissues was conducted. As shown in Fig. 7, *A–C*, gel-filtered bovine adrenal medulla cytosol contained several resolved functional activity peaks. Furthermore, priming activity peaks (Fig. 7 *B*) and triggering activity peaks (Fig. 7 *C*) did not co-elute. Priming activity was largely recovered in a peak at ~20 kD (Fig. 7 *B*, fractions 38 and 40), while the major peak of triggering activity was at 380 kD (fraction 14) with a minor peak of triggering activity at 37 kD (fraction 34). The results of Fig. 7, *A–C* indicated that in bovine adrenal medulla cytosol, partial reactions were stimulated by a limited number of factors which appeared to support either priming or triggering, but not both.

As shown in Fig. 7, *D–F*, the gel filtration profile for rat brain cytosol was somewhat more complex. Priming activity (Fig. 7 *E*) eluted in three peaks of ~500 (fractions 10–14), 120 (fraction 24), and 20 (fractions 38 and 40) kD. The elution of triggering activity (Fig. 7 *F*) in brain cytosol was similar to that of adrenal cytosol, except that an activity peak at 75 kD (fraction 28) was more prominent than the 37-kD (fraction 34) peak. With the exception of the highest molecular weight species, priming and triggering factors in brain cytosol eluted distinctively, and hence probably represent distinct proteins. The apparent multiplicity of priming factors in brain may account for the observation that brain cytosol was more effective in priming than adrenal cytosol (not shown).

It was of interest to determine whether the sequential action of two stage-specific factors could reconstitute Ca^{2+}-activated NE secretion in semi-intact PC12 cells. As shown in Fig. 8, sequential incubation with the 20-kD priming factor from adrenal (Fig. 7 *B*, fraction 40) and the 380-kD triggering factor from adrenal (Fig. 7 *C*, fraction 14) supported Ca^{2+}-activated NE secretion almost as fully as rat brain cytosol. However, when used in the reverse order, the stage-specific factors supported approximately as little secretion as expected for the complete omission of cytosolic proteins.

Preliminary Characterization of Priming and Triggering Factors from Rat Brain Cytosol

To further distinguish priming and triggering factors, we demonstrated that purification enriched for stage-specific activity. Priming activity in rat brain cytosol was fractionated by three chromatographic steps, resulting in a partially purified priming factor of 20 kD (see Materials and Meth-

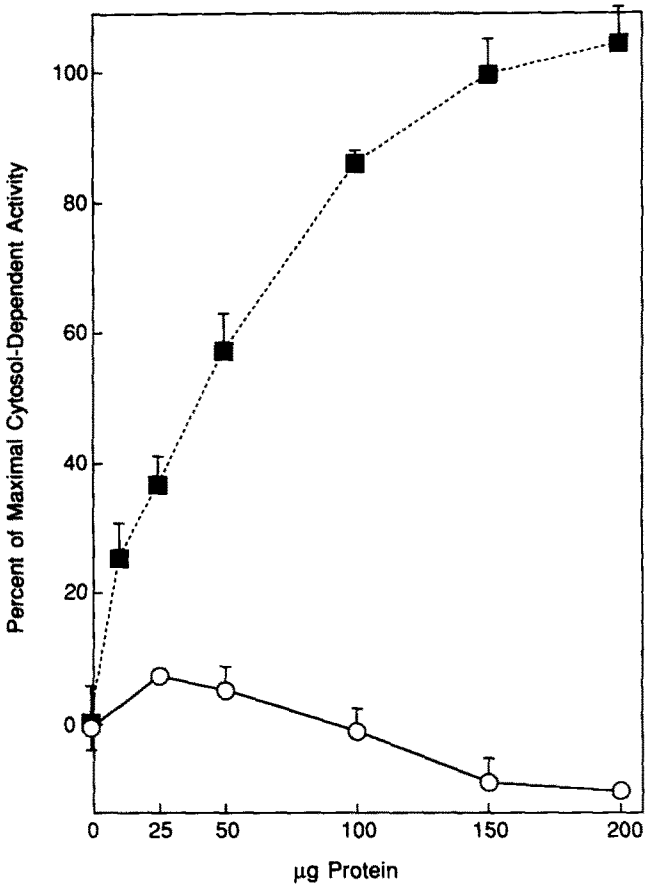

Figure 6. Cytosol priming and triggering activities have different thermolabilities. Rat brain cytosol was incubated at 46°C for 15 min to denature thermolabile proteins. Stimulation of NE secretion by the partially denatured cytosol was determined as a function of protein amount in priming (○) or triggering (■) incubations, using an optimally effective amount of untreated cytosol in the complementing partial reactions. Untreated cytosol was maximally effective in both priming and triggering reactions at 200 μg (not shown). For this experiment, inclusion of a saturating amount of control cytosol in both partial reactions resulted in 44% NE release; omission of cytosol from priming or triggering reactions resulted in 20 and 24% release, respectively.

ods). As shown in Fig. 9 *A*, this fraction contained priming activity (○) that was enriched relative to cytosol (compare Figs. 9 *A* and 5 *B*), but lacked significant triggering activity (■). Although the specific activity of this priming factor was increased relative to cytosol, the maximal activity had decreased. This could be explained by the fact that brain contains several priming factors, none of which alone provided the complete activity of cytosol (not shown).

p145, a novel 290-kD brain protein consisting largely of a dimer of 145-kD subunits has been purified based on its ability to reconstitute MgATP-dependent Ca^{2+}-activated NE secretion from semi-intact PC12 cells (Walent et al., 1992). A partially purified p145 fraction, corresponding to the third step in its purification (see Materials and Methods), was tested for priming and triggering activity. As shown in Fig. 9 *B*, the p145-containing fraction had maximal triggering activity (■) and virtually no priming activity (○). Furthermore, the triggering activity was enriched in comparison with cytosol (compare Figs. 9 *B* and 5 *B*). The triggering activity of p145 was only apparent when it was tested with primed semi-intact cells (not shown).

These results indicate that partially purified priming and triggering factors exhibit one but not both activities, reinforcing the conclusion that distinct cytosolic proteins mediate the two partial reactions.

Discussion

Resolution of Ca^{2+}-activated Secretion into Two Biochemically Distinguishable Partial Reactions

In these studies, MgATP-dependent Ca^{2+}-activated secretion from semi-intact PC12 cells was resolved into two distinct stages. The first stage, priming, was MgATP dependent and Ca^{2+} independent. The second stage, triggering, was Ca^{2+} dependent and MgATP independent. Since priming and triggering had distinct, nonoverlapping requirements for MgATP or Ca^{2+}, each partial reaction could be separately studied to identify additional molecular requirements. Priming and triggering may represent the production and consumption, respectively, of a key MgATP-dependent intermediate in the regulated secretory pathway. The intermediate is presumed to accumulate during priming because EGTA blocked its Ca^{2+}-dependent consumption. Addition of excess free Ca^{2+} during triggering would allow passage beyond the accumulation point to the completion of exocytosis.

Our results are consistent with several previous studies which indicated that ATP acts before Ca^{2+} in the regulated secretory pathway. A study with digitonin-permeabilized chromaffin cells (Holz et al., 1989) found that preincubations in the absence of Ca^{2+} resulted in the loss of a "primed" state required for rapid MgATP-independent secretion. Inclusion of MgATP in the preincubations partially maintained the primed state, perhaps by sustaining a labile MgATP-dependent intermediate. Howell et al. (1989) found that triggered exocytosis from streptolysin-*O*-permeabilized mast cells did not require ATP unless the cells were previously preincubated in its absence. Their interpretation was that ATP primed the response system, but was not required for Ca^{2+}- and GTPγS-triggered events. In sea urchin egg cortical fragments, ATP appeared to maintain secretory responsiveness, but was not required for Ca^{2+}-triggered exocytosis per se (Baker and Whitaker, 1978). In addition, it was recently reported that the presence of ATP during the isolation of *Paramecium* cortical preparations increased subsequent exocytotic responsiveness (Lumpert et al., 1990). This confirmed previous indications that ATP may have a priming role in trichocyst discharge (Vilmart-Seuwen et al., 1986).

An important point of agreement between our results and those mentioned above is the apparent lability of the MgATP-dependent intermediate. In each case, the primed state was readily lost upon incubation in the absence of ATP and Ca^{2+}; we termed this effect "depriming" (Fig. 4). Since the apparent loss of the primed state was itself reversed by priming, it seems likely that priming and depriming represent directly opposing reactions which generate or dissipate, respectively, the primed intermediate.

The major component of Ca^{2+}-activated secretion in semi-intact PC12 cells was found to require MgATP. The

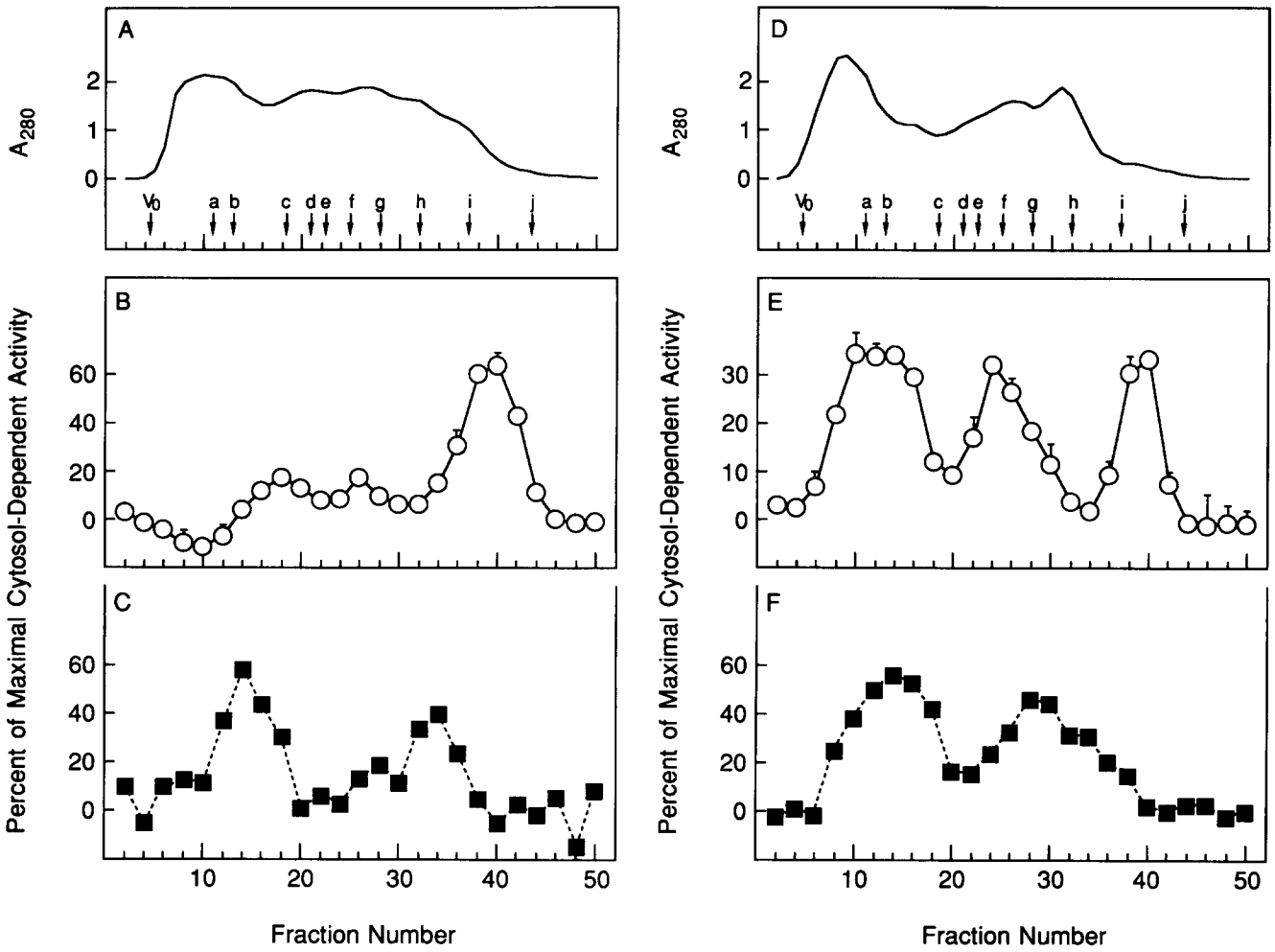

Figure 7. Resolution of cytosolic priming and triggering factors by gel filtration chromatography. Bovine adrenal medulla (*A–C*) and rat brain (*D–F*) cytosols were gel filtered, and individual column fractions were tested for UV absorbance (*A* and *D*), priming activity (*B* and *E*), and triggering activity (*C* and *F*). To test stimulation of NE secretion by column fractions, rat brain cytosol was used in the complementing partial reactions. The volumes of column fractions tested in priming and triggering incubations were 140 and 2 μl, respectively, for adrenal cytosol, and 25 and 25 μl, respectively, for brain cytosol. The distinct elutions of priming and triggering activities were evident over a wide range of tested volumes and degrees of stimulation (not shown). Elution positions of calibration markers are indicated by the arrows: V_0, dextran (2,000 kD); *a*, urease (540 kD); *b*, apoferritin (443 kD); *c*, β amylase (200 kD); *d*, catalase (240 kD); *e*, alcohol dehydrogenase (150 kD); *f*, phosphorylase B (97 kD); *g*, BSA (68 kD); *h*, ovalbumin (45 kD); *i*, carbonic anhydrase (29 kD); and *j*, cytochrome C (12.4 kD). For the data shown in *B* and *C*, inclusion of a saturating amount of control cytosol in both partial reactions resulted in 50% NE release; omission of cytosol from priming or triggering reactions resulted in 21 and 32% release, respectively. For the experiment shown in *E* and *F*, the respective release values were 58, 26, and 34%.

MgATP requirement appeared to reside exclusively with the priming reaction, as demonstrated using hexokinase (Fig. 3). However, we cannot exclude the possibility that MgATP has additional roles in secretion which are not evident under the conditions of either partial reaction. For example, an additional event might occur before or concomitant with Ca^{2+}-triggered exocytosis, and require both Ca^{2+} and ATP. We were unable to fully evaluate this latter possibility. Ca^{2+}-activated secretion from fully primed semi-intact cells was stimulated to a small extent by inclusion of MgATP (Fig. 1). This might result either from an additional late-stage role for MgATP, or from continuing priming which counteracts the depriming that inevitably occurs during triggering incubations in the absence of MgATP.

Distinct Cytosolic Proteins Mediate Sequential Partial Reactions

Although previous studies provided evidence that ATP acts before Ca^{2+} in the regulated secretory pathway (see above), they did not identify a requirement for cytoplasmic proteins in these sequential steps. In contrast, we were able to identify soluble protein requirements for the distinct MgATP-dependent priming and Ca^{2+}-triggered steps. Unlike permeabilization by other methods, "cell cracking" allows the relatively complete depletion and reconstitution of cells with soluble cytoplasmic molecules (Martin, 1989; Martin and Walent, 1989). Since we were able to deplete the semi-intact cells of soluble proteins before both partial reactions, the re-

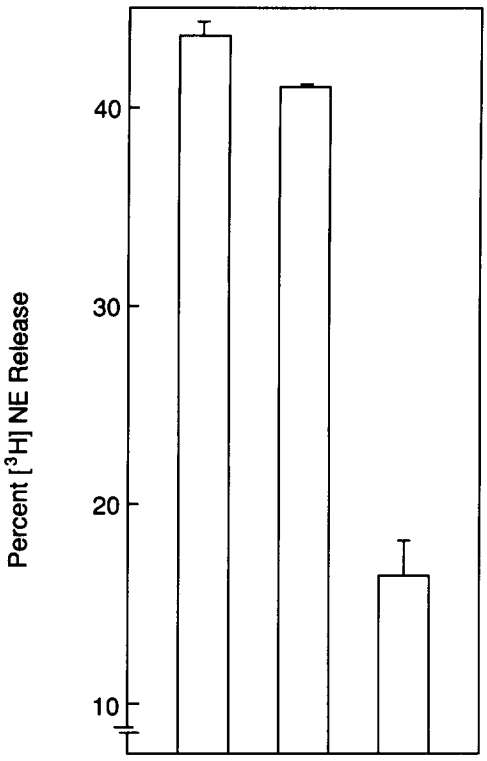

Figure 8. Two stage-specific cytosol fractions can effectively reconstitute priming and triggering. Priming and triggering incubations were carried out using either unfractionated rat brain cytosol or bovine adrenal fractions which possessed stage-specific activity in the experiment shown in Fig. 7, *A–C*. NE secretion was determined using the following combinations of cytosol fractions: first bar, saturating rat brain cytosol in both partial reactions; second bar, 140 μl of fraction 40 for priming and 50 μl of fraction 14 for triggering; third bar, 140 μl of fraction 14 for priming and 50 μl of fraction 40 for triggering.

quirements for the reconstitution of both steps could be characterized.

The results established several important features of the requirements for cytosolic proteins in priming and triggering. First, both priming and triggering were dependent upon or stimulated by cytosolic proteins (Fig. 5). Triggering appeared to depend less completely upon cytosol factors than priming. The basis for such differences is unclear at the present time. Triggering may be partially supported by the activity of membrane- or cytoskeletal-associated counterparts to the identified cytosolic triggering factors.

Second, the priming and triggering activities of cytosol were functionally distinct and mediated by separate proteins. Since priming and triggering conditions differed with respect to MgATP and Ca^{2+}, it seemed likely that the cytosolic factors involved in each step participate in different types of biochemical reactions. The distinctness of cytosolic priming and triggering activities was indicated by their differential thermolability (Fig. 6). Moreover, stage-specific factors in cytosol were resolved chromatographically (Fig. 7), indicating that priming and triggering activities reside with distinct proteins. Two partially purified brain factors were shown to be enriched for stage-specific activity, one for priming, the other for triggering (Fig. 9). The fact that priming and triggering factors are distinct suggests the possibility that these factors participate directly in the distinct MgATP- and Ca^{2+}-dependent events.

Third, a limited number of cytosolic factors appeared to support the partial reactions. Chromatographic profiles of cytosolic priming and triggering factors were about as complex as those of intra-Golgi transport factors (Wattenberg and Rothman, 1986). Gel-filtered rat brain cytosol contained three priming factor peaks with equal activity (Fig. 7 *E*), and three triggering factor peaks, two of which were not well resolved (Fig. 7 *F*). Bovine adrenal medulla cytosol was less complex, with only one major priming and two major triggering factor peaks (Fig. 7, *B* and *C*).

This work classifies the active components in cytosol into two categories, priming factors and triggering factors, the sequential actions of which are sufficient to reconstitute MgATP-dependent Ca^{2+}-activated secretion. Although two stage-specific factors were sufficient to support regulated secretion (Fig. 8), several peaks of each type of stage-specific activity were resolved by gel filtration. The multiple peaks for a given activity may represent distinct but functionally redundant cytosolic proteins. Alternatively, the multiplicity of peaks for a given activity may be explained by complexes of a single active species with other cytosolic proteins. However, we cannot eliminate the possibility that multiple peaks of activity arose artifactually as the result of proteolysis, aggregation, anomalous gel filtration, or overlapped elution with inhibitory factors.

It is possible that one or more essential cytosolic proteins were not detected in our studies, since regulated secretion in semi-intact cells may be supported in part by cytoskeletal or membrane-associated counterparts to proteins which also exist in the cytosol. Hence, the back addition of certain essential cytosolic proteins may not be rate limiting, and these proteins would not be detected in either partial reaction.

Molecular Identities of Priming and Triggering Factors

The molecular identities of the soluble proteins which mediate priming and triggering events have not been fully established. Future studies should clarify the relationship between these proteins and several cytoplasmic (or partially cytoplasmic) proteins previously implicated in secretion (reviewed by Burgoyne, 1991). Addition of calpactin to digitonin-permeabilized chromaffin cells was reported to retard the loss of secretory responsiveness accompanying the gradual washout of cytoplasmic protein^{-} (Ali et al., 1989). In the present study, a 37-kD triggering factor peak (most prominent in adrenal; Fig. 7 *C*, fraction 34) was identified which is similar in size to calpactin heavy chain, p36. However, it is unlikely that this peak corresponds to p36, since purified lung calpactin or p36 do not stimulate priming, triggering, or overall secretion in semi-intact PC12 cells (T. F. J. Martin and J. Kowalchyk, unpublished observations). The relevance of calpactin to the functional activity of cytosol has also recently been questioned by other studies (Wu and Wagner, 1991; Walent et al., 1992).

Recently a ~30-kD protein from brain cytosol was purified based upon its ability to retard secretory run-down

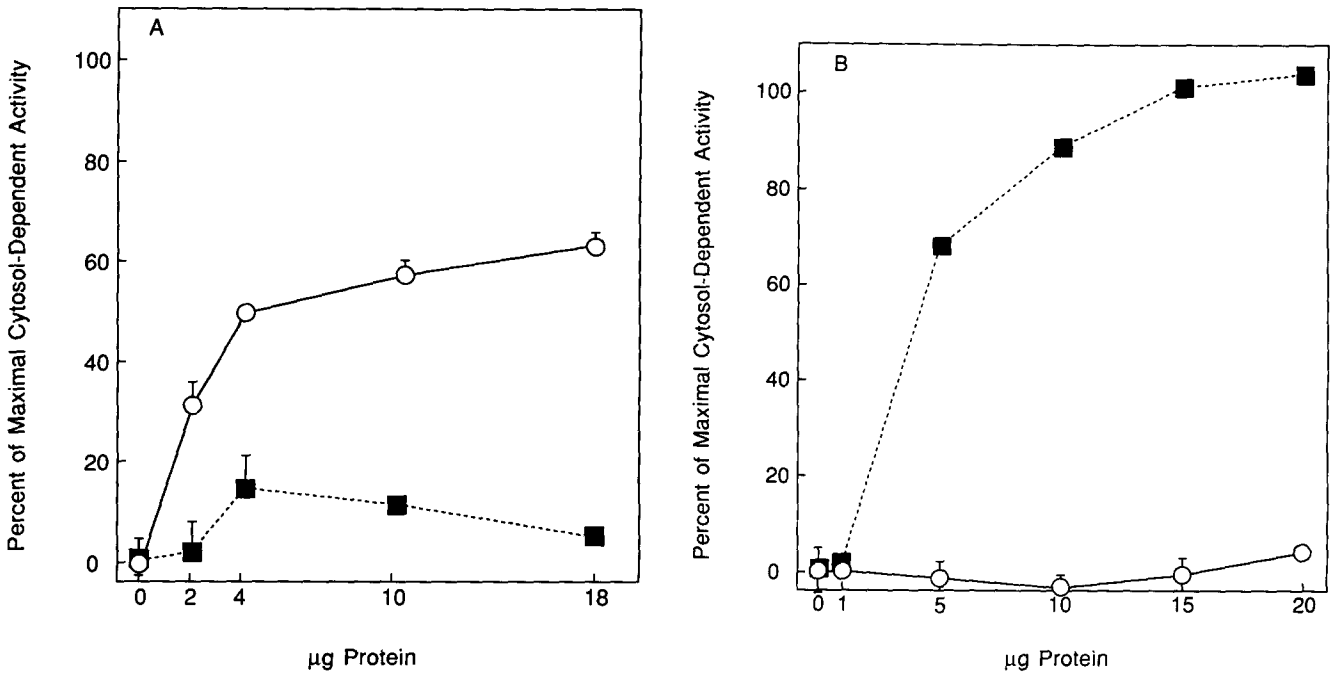

Figure 9. Partially purified cytosolic factors are enriched for stage-specific activity. *A* shows stimulation of NE secretion by a 20-kD priming factor as a function of protein amount in priming (○) or triggering (■) incubations, using a maximally effective amount of fraction 14 (Fig. 7 *C*) or rat brain cytosol, respectively, in the complementing partial reactions. *B* shows stimulation of NE secretion by partially purified p145 as a function of protein amount in priming (○) or triggering (■) incubations, using a maximally effective amount of rat brain cytosol in the complementing partial reactions. For the experiment in *A*, inclusion of a saturating amount of control cytosol in both partial reactions resulted in 52% NE release; omission of cytosol from priming or triggering reactions resulted in 22 and 32% release, respectively. For the experiment shown in *B*, the respective release values were 51, 18, and 30%.

in digitonin-permeabilized chromaffin cells (Morgan and Burgoyne, 1992). The role of this protein, termed Exo1, and an additional unpurified component, termed Exo2, in Ca^{2+}-activated secretion remains unknown. Gel filtration of Exo1 and Exo2 suggested native sizes of 70 and 44 kD, respectively (Morgan and Burgoyne, 1992), which are perhaps compatible with triggering activity peaks at 75 and 37 kD (Fig. 7, *C* and *F*, fractions 28 and 34). However, Morgan and Burgoyne (1992) reported that the ability of cytosol (and presumably the Exo proteins) to retard secretory run-down was *N*-ethylmaleimide (NEM) sensitive. In contrast, the priming and triggering activities of cytosol and all cytosolic factors detected in this work were NEM-insensitive (not shown; 10 mM NEM, 15 min, 0°C). More specific reagents will be required to establish the relationship of Exo1 and Exo2 to priming and triggering factors.

One described protein whose role in the partial reactions appears established is p145, purified from rat brain cytosol based on its ability to reconstitute MgATP-dependent Ca^{2+}-activated NE secretion from semi-intact PC12 cells (Walent et al., 1992). Native p145 consists largely of a ~290-kD dimer of 145-kD subunits, but trimers and tetramers are also present; these species are not resolved by Superose 12 gel filtration. A partially purified brain p145 fraction was found to be enriched for triggering activity and devoid of priming activity (Fig. 9 *B*). Furthermore, the high molecular weight (~380 kD) triggering factor detected in gel-filtered cytosol (Fig. 7, *C* and *F*, fraction 14) co-eluted with p145 immunoreactivity (not shown), indicating that p145 and the high molecular weight triggering factor are likely identical. p145 was previously shown to be the major cytosolic factor responsible for reconstituting regulated secretion in semi-intact PC12 cells (Walent et al., 1992) and the present results suggest that it is the major triggering factor.

Previous studies have implicated protein phosphorylation in the control of Ca^{2+}-activated secretion (Lee and Holz, 1986; Wagner and Vu, 1989, 1990). MgATP-dependent priming might consist of protein phosphorylation which regulates the activity of key membrane or cytoskeletal proteins involved in secretory granule transport, targeting, or fusion. The results with several general protein kinase inhibitors (Table I) are compatible with a requirement for protein phosphorylation in priming. A major limitation of the inhibitor data, however, is the possibility that the inhibitors affect other ATP-dependent proteins by virtue of their action at ATP-binding sites. One class of ATP-dependent proteins which might be inhibited by the drugs, and also may have a required role in MgATP-dependent Ca^{2+}-activated secretion, are the phosphoinositide kinases. A correlation between polyphosphoinositide levels and the primed state has been reported (Eberhard et al., 1990). Priming could represent an increase in the abundance of polyphosphoinositides required to mediate associations between membranes and key fusion proteins. A role for other ATP-dependent enzymes in priming cannot be eliminated by the existing data.

Protein kinase C is commonly cited for its stimulatory influence on Ca^{2+}-activated secretion in diverse systems (Kikkawa and Nishizuka, 1986; Ben-Shlomo et al., 1991). Wagner and Vu (1989; 1990) provided evidence that MgATP-dependent Ca^{2+}-activated NE secretion in digitonin-permeabilized PC12 cells may be regulated by an unspecified Ca^{2+}-dependent protein kinase. We note that protein kinase

C, or any other Ca^{2+}-dependent protein kinase, seems unlikely to be an essential requirement for Ca^{2+}-activated secretion since the reaction conditions for priming and triggering exclude an activity which is both ATP and Ca^{2+} dependent. However, recent studies have identified a modulatory role for protein kinase C since this enzyme does appear to regulate the activity of essential proteins required for triggering and possibly priming (Nishizaki et al., 1992; Walent et al., 1992).

An unidentified GTP-binding protein has been implicated in the control of regulated exocytosis based upon the striking stimulatory effects of GTP analogs in permeabilized cells (reviewed by Gomperts, 1990). A requirement for small molecular weight GTP-binding proteins has been well established for earlier steps of the secretory pathway (reviewed by Rothman and Orci, 1992), and for the fusion of constitutive vesicles with the plasma membrane (Salminen and Novick, 1987). In light of the apparent universality of GTP-binding proteins in membrane trafficking events, it seemed plausible that one or more of the soluble factors detected in the present study may be a GTP-binding protein or a protein which regulates their activity. However, we have been unable to obtain evidence for the involvement of a GTP-binding protein in priming or triggering using GTP or its analogs (not shown). The function of GTP-binding proteins in this system may require less than one full cycle of guanine nucleotide exchange and hydrolysis, and sufficient GTP may be present endogenously in semi-intact cells or added cytosolic proteins to preclude detecting a requirement for the nucleotide. Alternatively, the existence of a bona fide ATP-dependent step along with the capacity to generate GTP might mask a GTP corequirement in this system.

Relationship of Priming and Triggering Factors to Components of the Constitutive Secretory Pathway

It is not known to what degree, if any, the events and components required for the regulated pathway are similar to those for constitutive exocytosis. Post-Golgi events in the constitutive pathway are ATP and cytosol dependent but do not appear to require intracellular Ca^{2+} (Helms et al., 1990; Miller and Moore, 1991). Since MgATP-dependent priming is independent of Ca^{2+}-triggered events, the priming factors identified by our assay might also function in constitutive exocytosis. Although hepatic cells exhibit a highly active constitutive secretory pathway, the secretion of liver proteins is not known to be Ca^{2+} regulated. Nevertheless, liver cytosol contains a priming factor with chromatographic properties very similar to those of the 20-kD factor in brain and adrenal medulla, consistent with a possible role in the constitutive pathway (our own unpublished observations). Further studies will be required to determine whether the cytosolic factors mediating priming in regulated systems have a similar function in constitutive secretion.

Cytosolic proteins which support Ca^{2+} triggering seem less likely to perform a common function in both regulated and constitutive secretion, since their stage-specific function requires elevated intracellular Ca^{2+}. Furthermore, the expression of the p145 protein, apparently the major cytosol factor mediating Ca^{2+} triggering, is limited to tissues specialized for regulated secretion (Walent et al., 1992).

We have dissected the process of MgATP-dependent Ca^{2+}-activated secretion into two sequential partial reactions, each involving a subset of the biochemical events required for regulated secretion. Further characterization of the soluble proteins which mediate the partial reactions should help to clarify the nature of these events.

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