Abstract. We have used stage-specific assays for MgATP-dependent priming and for Ca\textsuperscript{2+}-activated triggering in the absence of free MgATP to examine the effects of α-SNAP, 14-3-3 proteins and calmodulin on regulated exocytosis in permeabilized adrenal chromaffin cells. All three proteins lead to a Ca\textsuperscript{2+}-dependent increase in catecholamine secretion. Both α-SNAP and 14-3-3 proteins stimulated in a priming but not in a triggering assay. In contrast, calmodulin was stimulatory in triggering but not priming. The effects of α-SNAP and 14-3-3 proteins were likely to be due to distinct mechanisms of action since they differed in Ca\textsuperscript{2+}-dependency, time course and extent of stimulation and their effects were additive. α-SNAP and 14-3-3 proteins did not appear to exert their priming action through changes in synthesis of phosphatidylinositol (4,5) bisphosphate. The data show that these three proteins have distinct stage-specific actions on exocytosis and indicate that α-SNAP acts in an early MgATP-requiring stage and not in the late Ca\textsuperscript{2+}-triggered steps immediately prior to membrane fusion as previously suggested.
proteins syntaxin and SNAP-25 (collectively called the SNAREs). Exocytosis would be triggered by α-SNAP binding to the SNAREs, followed by NSF binding to α-SNAP and ATP-hydrolysis by NSF. NSF would cause reorganization of the docking (SNARE) complex and allow membrane fusion to occur. This model places ATP hydrolysis as a late step immediately preceding membrane fusion. However, as noted above Ca\(^{2+}\) can trigger exocytosis in several cell types in the absence of free MgATP which is not in agreement with the model of Söllner et al. (1993a).

From the use of clostridial neurotoxins, the essential nature of synaptobrevin, syntaxin, and SNAP-25 for exocytosis in neurons and neuroendocrine cells seems to be established since cleavage of these proteins by the neurotoxins blocks exocytosis (Blasi et al., 1993; Schiavo et al., 1992, 1993). Adrenal chromaffin cells express the SNARE proteins which can be isolated in a SNARE complex (Hodel et al., 1994; Roth and Burgoyne, 1994) and exocytosis can be stimulated by introduction of exogenous α-SNAP into digoxigenin-permeabilized chromaffin cells (Morgan and Burgoyne, 1995). We have, therefore, used stage-specific assays for exocytosis in chromaffin cells in order to examine where α-SNAP acts in exocytosis and to compare its actions to two other exocytosis-stimulating factors, the 14-3-3 proteins and calmodulin. The data obtained suggested that whereas calmodulin acts to stimulate the triggering reaction, both α-SNAP and 14-3-3 proteins act in the earlier MgATP-dependent priming stage but appear to have distinct actions.

**Materials and Methods**

**Materials**

High purity digoxigenin was obtained from Novabiochem (Nottingham, UK). Fetal calf serum and Dulbecco’s modified Eagle’s medium with 25 mM Hepes were obtained from Gibco (Paisley, UK). All other reagents were of analytical grade from Sigma (Poole, UK). KGEP buffer contained 139 mM potassium glutamate, 20 mM Pipes, and 5 mM EGTA (pH 6.5). KGEP/MgATP buffer also contained 2 mM ATP and 2 mM MgCl\(_2\). Permeabilization buffer was KGE with 2 mM ATP, 2 mM MgCl\(_2\), and 20 μM α-digitoxin digoxigenin. The published protocol for the purification of Exol (Morgan and Burgoyne, 1992a) was used for the purification of 14-3-3 proteins from sheep brain cytosol by ion-exchange, hydrophobic interaction, and Mono Q fast protein liquid chromatography (f.p.l.c.) followed by gel filtration. Expression and purification of recombinant α-SNAP was based on a previously published method using a plasmid encoding α-SNAP and purification of the His\(_6\)-tagged protein on Ni-NTA-agarose (Whiteheart et al., 1993).

**Isolation and Culture of Chromaffin cells**

Chromaffin cells were isolated from bovine adrenal medulIae by enzymic digestion as described by Greenberg and Zinder (1982) with modifications (Burgoyne et al., 1988). Cells were washed in calcium-free Krebs-Ringer buffer, consisting of 145 mM NaCl, 5 mM KCl, 1.3 mM MgCl\(_2\), 1.2 mM NaH\(_2\)PO\(_4\), 10 mM glucose, and 20 mM Hepes at pH 7.4, resuspended in culture medium (Dulbecco’s modified Eagle’s medium with 25 mM Hepes, 10% fetal calf serum, 8 μM fluoride, 50 μg/ml gentamycin, 10 μg/ml cyclosporin A, 2.5 μg/ml amphotericin B. 25 U/ml penicillin, 25 μg/ml streptomycin), plated in 24-well trays at a density of one million cells per well and maintained in culture for 3–7 d before use. In later experiments amphotericin B was omitted from the media.

**Preparation of Cytosol**

Brains were extracted from Wistar rats and homogenized in ice-cold homogenization buffer, consisting of 20 mM Tris, 1 M KCl, 250 mM sucrose, 2 mM MgCl\(_2\), 2 mM ATP, 1 mM DTT, 1 mM EGTA, and 1 mM PMSF (pH 8.0). The homogenized brains were subsequently centrifuged for 60 min at 100,000 g (4°C) to remove cell debris and the recovered cytosol was dialyzed overnight against KGE/MgATP buffer (see Materials and Methods). The dialyzed cytosol was centrifuged for 60 min at 100,000 g and was stored at -20°C.

**Cell Permeabilization and Assay of Catecholamine Secretion**

After washing each well with 1 ml of calcium-free Krebs-Ringer buffer, the cultured cells were permeabilized by incubation with 300 μM of permeabilization buffer. After permeabilization, catecholamine release was examined under different conditions as described in the figure legends. However, the priming, triggering, and combined priming and triggering assays which were developed are outlined below:

- **Combined Assay.** Step 1. Incubation with permeabilization buffer for 45 min. Step 2. Incubation with KGE/MgATP buffer and various free Ca\(^{2+}\) concentrations (0–10 μM) for 15 min. Purified proteins, cytosol, or dialysis buffer (control) were included in step 2 as indicated. Catecholamine release during this step was assayed.

- **Priming Assay.** Step 1. Incubation with permeabilization buffer for 45 min. Step 2. Incubation with KGE/MgATP buffer and various free Ca\(^{2+}\) concentrations (0–10 μM) for 15 min. Purified protein, cytosol, or dialysis buffer was also included in step 2 as indicated. Catecholamine release during step 2 was assayed.

- **Triggering Assay.** Step 1. Incubation with permeabilization buffer for 25 min. Step 2. Incubation with KGE/MgATP buffer (i.e., with no MgATP) with various Ca\(^{2+}\) concentrations (0–10 μM) for 15 min. Purified protein, cytosol, or dialysis buffer was also included in step 2 as indicated. Catecholamine release during step 2 was assayed.

Assay of released catecholamines was performed using a fluorometric method (von Euler and Flodin, 1955). Total catecholamine content of the cells was determined after release of catecholamines with 1% Triton-X-100 and catecholamine secretion was calculated as a percentage of the total cellular catecholamine. All experiments were performed at room temperature (20–22°C). All results were confirmed in experiments on at least three separate batches of cells.

**Assay of Phosphatidylinositol (4,5) Bisphosphate**

Cells were incubated for 45 min in permeabilization buffer and then for 15 min with 0 or 10 μM Ca\(^{2+}\) in the absence or presence of proteins. During the latter period, 5 μCi [\(^{3}P\)]-ATP (specific activity 3 Ci/mmol; Amersham) per well (300 μl) was present. After the 15 min stimulation period, the supernatant was removed, and lipids extracted as described by Eberhard and Holz (1991). In brief, cells were solubilized in 100 μl of ice-cold methanol /hydrochloric acid (100:1, vol/vol), scraped off the wells, and washed with another 100 μl of methanol/HCl as above. Pooled samples were extracted with 400 μl of chloroform/methanol (2:1), vortexed, and 250 μl of an EDTA/HCl solution was added (10 mM EDTA, 1 M HCl). Samples were vortexed and centrifuged for 30 s at 14,500 g. 200 μl of the lower organic phase was transferred to a new microfuge tube. The upper phase was re-extracted by a further addition of 200 μl chloroform/methanol (2:1) and the organic phase was removed after vortexing and centrifugation. Pooled organic phases were washed with 400 μl of ethylacetate (2:1) and the aqueous phase was removed after vortexing. Lipids in the organic phase were dried under nitrogen and re-dissolved in 15 μl of chloroform. Samples were spotted onto thin layer chromatography (t.l.c.) plates (silica 60; Merck, Sharpe, & Dohme, Rahway, NJ) that had been impregnated with 1% potassium oxalate and baked at 120°C for 1 h. Any remaining lipids were rinsed off the microfuge tubes by addition of a further 7 μl of chloroform, that was spotted onto the same t.l.c. plate. The t.l.c. was run with chloroform/methanol/concentrated ammonia (35%)/H\(_2\)O (90:90:7:20) as a mobile phase in a saturated chamber. Radioactive phospholipids were visualized with a Molecular Dynamics

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1. Abbreviations used in this paper: FPLC, fast protein liquid chromatography; NSF, N-ethylmaleimide-sensitive fusion protein; NTA, nitrotriacetic acid; PtdIns(4,5)P\(_2\), phosphatidylinositol (4,5) bisphosphate; SNAP-25, synaptosomal associated protein of 25 KD; SNAREs, soluble NSF attachment proteins; SNAREs, SNAP receptors; t.l.c., thin layer chromatography.
Results

MgATP-dependent and -independent Secretion from Digitonin-permeabilized Chromaffin Cells

Optimal secretion from digitonin-permeabilized chromaffin cells occurs in the presence of MgATP but a significant component of secretion is detectable in the absence of free MgATP (Holz et al, 1989). In our study, MgATP-dependent secretion was half maximal at around 400 μM MgATP (not shown). MgATP-independent release was Ca²⁺-dependent and was largely complete within 8 min. This MgATP-independent secretion could still be detected, albeit at a lesser extent, after prolonged permeabilization (>45 min) in the presence of MgATP but the secretory responsiveness of the cells when challenged with Ca²⁺ in the presence or absence of MgATP was rapidly and irreversibly lost if the cells were permeabilized and maintained in the absence of MgATP prior to stimulation (data not shown). During a 45-min permeabilization period in the presence of MgATP a general run-down of responsiveness of permeabilized chromaffin cells occurs. In order to examine the effects of cytosolic proteins on priming, an assay was designed in which cells were permeabilized for 45 min, incubated with added cytosolic proteins in the presence of free MgATP for 15 min, and then release due to 10 μM Ca²⁺ in the absence of MgATP was measured in a 4-min incubation. The time of the final incubation was chosen as a time at which MgATP-independent secretion was still proceeding at close to the maximal rate as it has been shown that the major effect of priming in PC12 cells is to increase the initial rate of exocytosis (Hay and Martin, 1992). Priming in permeabilized chromaffin cells after short permeabilization times (prior to extensive loss of cytosolic proteins) is enhanced at intermediate (1 μM) Ca²⁺ concentrations (Bittner and Holz, 1992). In contrast, cytosolic priming factors identified from studies on PC12 cells did not require elevated Ca²⁺ for activity (Hay and Martin, 1992, 1993). In our initial experiments it was found that brain cytosol stimulated priming at 0 Ca²⁺ and additionally showed a Ca²⁺-dependent effect maximal at 1 μM Ca²⁺ (data not shown). It is probable, therefore, that cytosol contains multiple priming factors that differ in their requirement for Ca²⁺.

Effects of α-SNAP, 14-3-3 Proteins and Calmodulin in Combined and Priming Assays

The purity of α-SNAP, 14-3-3 proteins and calmodulin used in these studies was confirmed by SDS–polyacrylamide gel electrophoresis (Fig. 1). Each protein was tested at concentrations previously determined to be maximal. When cells were stimulated with Ca²⁺ in the presence of MgATP (the combined assay since both priming and triggering can occur) each of the proteins increased secretion in a Ca²⁺-dependent manner (Fig. 2) as expected from the previous work on α-SNAP (Morgan and Burgoyne, 1995), 14-3-3 proteins (Morgan and Burgoyne, 1992a) and calmodulin (Okabe et al, 1992). The extent of the stimulation by α-SNAP was smaller than that seen with 14-3-3 proteins or calmodulin in this standard assay with a 15-min stimulation period but with longer stimulation times the effect of α-SNAP was more marked (see below). In addition, the stimulatory effect of α-SNAP in this assay was only seen at a Ca²⁺ concentration of 10 μM whereas that due to 14-3-3 proteins and calmodulin was seen at lower Ca²⁺ concentrations.

The effect of these proteins on MgATP-dependent priming was also examined. The data in Fig. 3 shows secretion in response to a 10 μM Ca²⁺ challenge in the absence of MgATP after incubation with the proteins at various Ca²⁺ concentrations in a prior priming step in the presence of MgATP. When the proteins were included in the priming assay both α-SNAP and 14-3-3 proteins stimulated priming but calmodulin did not (Fig. 3). The stimulatory effect of α-SNAP was maximal when the priming step contained 10 μM Ca²⁺ whereas the stimulatory effect of 14-3-3 pro-
Calmodulin did not stimulate in the priming assay. Chromaffin cells were permeabilized for 45 min and incubated for 15 min in 200 μl KGEP/MgATP buffer with the indicated free Ca^{2+} concentration in the presence or absence of 25 μg/ml α-SNAP (A), 250 μg/ml 14-3-3 proteins (B), or 250 μg/ml calmodulin (C). The cells were then incubated in 300 μl KGEP buffer containing 10 μM Ca^{2+} for 4 min and catecholamine release during this last incubation was assayed. Data shown are means ± S.E. (n = 4) and are expressed as percentage of total cellular catecholamine at the beginning of the release incubation. Note that this data shows the Ca^{2+}-dependence of the priming reaction.

**Figure 3.** Effects of α-SNAP, 14-3-3 proteins and calmodulin on priming of Ca^{2+}-activated secretion from permeabilized chromaffin cells. Cells were permeabilized for 45 min. The cells were then incubated for 15 min in 200 μl KGEP/MgATP buffer with the indicated free Ca^{2+} concentration in the presence or absence of 25 μg/ml α-SNAP (A), 250 μg/ml 14-3-3 proteins (B), or 250 μg/ml calmodulin (C). The cells were then incubated in 300 μl KGEP buffer containing 10 μM Ca^{2+} for 4 min and catecholamine release during this last incubation was assayed. Data shown are means ± S.E. (n = 4) and are expressed as percentage of total cellular catecholamine at the beginning of the release incubation. Note that this data shows the Ca^{2+}-dependence of the priming reaction.

**Figure 4.** Effects of combinations of α-SNAP, 14-3-3 proteins and calmodulin in the priming reaction. Chromaffin cells were permeabilized for 45 min and incubated for 15 min in 200 μl per well KGEP/MgATP containing 10 μM Ca^{2+} with or without α-SNAP (25 μg/ml), 14-3-3 proteins (250 μg/ml), or calmodulin (250 μg/ml) as indicated. The cells were then incubated in 300 μl per well KGEP buffer with 10 μM Ca^{2+} for 4 min and catecholamine release determined. Data shown are means ± S.E. (n = 4) and are expressed as percentage of total cellular catecholamine at the beginning of the release incubation. Calmodulin did not stimulate priming either alone or in combination with other proteins. The stimulatory effects of α-SNAP and 14-3-3 proteins on priming were additive.

**Effect of α-SNAP, 14-3-3 Proteins, and Calmodulin in a Triggering Assay**

Since calmodulin did not stimulate in the priming assay despite stimulating secretion in a combined assay we examined the effect of calmodulin in an assay designed to measure stimulation of the triggering reaction. In this assay the cells were stimulated with Ca^{2+} with or without added proteins in the absence of MgATP. Under these conditions the protein would be unable to stimulate MgATP-dependent priming and any stimulation would thus be due to an effect on the Ca^{2+}-triggering reaction. As shown in Fig. 6 C, calmodulin gave a Ca^{2+}-dependent increase in secretion compared to controls. In contrast, α-SNAP and 14-3-3 proteins had no stimulatory effect in the triggering assay and were to some extent inhibitory (Fig. 6, A and B). The differing effects of calmodulin compared to α-SNAP and 14-3-3 proteins in the two assays provides confirmation that the assays can distinguish between proteins with different mechanisms of action.

**Effect of Cytosol, α-SNAP, and 14-3-3 Proteins on PtdIns(4,5)P_2 Synthesis**

It has been suggested that MgATP-dependent priming in permeabilized chromaffin cells may, in part, be related to polyphosphoinositide synthesis (Eberhard and Holz, 1990; Eberhard et al., 1991). Recent work on permeabilized PC12 cells has identified three cytosolic factors active in a priming assay similar to that used here (Hay and Martin,
Discussion

It has previously been shown that Ca\(^{2+}\)-dependent exocytosis in permeabilized chromaffin cells is stimulated by α-SNAP (Morgan and Burgoyne, 1995) and by 14-3-3 proteins (Morgan and Burgoyne, 1992a; Roth et al., 1994). In the protocol used previously cells were permeabilized, preincubated with exogenous proteins and then stimulated by Ca\(^{2+}\) in the presence of MgATP in a subsequent step. This assay would not allow assessment of whether these proteins act in priming or triggering stages of exocytosis. In addition, calmodulin has been shown to stimulate exocytosis in permeabilized chromaffin cells but its stage of action is unknown (Okabe et al., 1992).

We have now used stage-specific assays for ATP-dependent priming and Ca\(^{2+}\)-mediated triggering of exocytosis in permeabilized adrenal chromaffin cells to determine which stages are stimulated by the three proteins, α-SNAP, 14-3-3 proteins, and calmodulin. The assays were based on those previously used and extensively characterised with digitonin-permeabilized chromaffin cells (Bittner and Holz, 1992; Holz et al. 1989) and with PC12 cells permeabilized using a ball homogenizer (Hay and Martin, 1992, 1993). It was shown that distinct cytosolic factors activated each of the stages in PC12 cells but the effects of cytosolic factors on priming and triggering in chromaffin cells have not been examined. From the studies on PC12 cells, p145 was shown to be a triggering factor (Hay and Martin, 1992; Walent et al., 1992) and three priming factors have been identified (Hay and Martin, 1992). The effect of these factors on priming did not require the presence of elevated Ca\(^{2+}\) during the priming reaction but priming in chromaffin cells is Ca\(^{2+}\)-dependent (Bittner and Holz, 1992). Using these stage-specific assays we found distinct actions of α-SNAP, 14-3-3 proteins, and calmodulin. Calmodulin acted in the triggering but not the priming assay whereas α-SNAP and 14-3-3 proteins stimulated only in the ATP-dependent priming assay. The effects of all three proteins were Ca\(^{2+}\)-dependent. The validity of the assays used was supported by the finding that purified factors could act in either priming or triggering reactions but not in both.

The effects of α-SNAP and 14-3-3 proteins were additive and showed different Ca\(^{2+}\)-dependencies and time courses suggesting that they had distinct actions in priming. Priming is a general term given for ATP-dependent events that occur prior to membrane fusion which are likely to include preparation of the exocytotic machinery for fusion or regulation of secretory vesicle availability prior to vesicle docking. It is likely that priming includes...
The possible role of calmodulin in exocytosis has been discussed extensively but remains controversial. While earlier data suggested that calmodulin might be involved in exocytosis in chromaffin cells (Kenigsberg and Trifaro, 1985) other studies suggested that calmodulin was not an essential component (Brooks and Treml, 1984). In contrast, calmodulin is an essential requirement for exocytosis in Paramecium (Kerboeuf et al., 1993). Okabe et al. (1992) found that exogenous calmodulin stimulates exocytosis in permeabilized chromaffin cells and we have been able to confirm that finding and extend it to show that calmodulin acts in an ATP-independent step. The effect of calmodulin on exocytosis was more pronounced in the combined (Fig. 2) compared to the triggering (Fig. 6) assay. This difference may be due to the fact that the combined assay mea-
sures secretion over 15 min and allows repeated cycles of priming and triggering to occur with multiple rounds of triggering for calmodulin to act upon, whereas the triggering assay allows only a single burst of triggered exocytosis of those vesicles already primed for release. Its site of action is unclear but an action mediated via calmodulin-dependent protein kinases is unlikely due to its lack of requirement for MgATP. One possible target is the vesicle protein synaptotagmin, which is a component of the SNARE complex (Sollner et al., 1993a), which may be a Ca2+ receptor in exocytosis (Geppert et al., 1994) and is a known calmodulin-binding protein (Fournier and Trifaro, 1988; Tugal et al., 1991). The ability of synaptotagmin to bind calmodulin is controversial and calmodulin may have an alternative mechanism of action.

In conclusion, we have been able to define distinct stage-specific actions of α-SNAP, 14-3-3 proteins, and calmodulin in exocytosis in permeabilized chromaffin cells. These data provide further insight into the action of 14-3-3 proteins and calmodulin in exocytosis but a key finding of the study is that α-SNAP acts in an early ATP-dependent priming stage. The latter finding is not consistent with the proposed role of α-SNAP in regulated exocytosis (Sollner et al., 1993a) and suggests that α-SNAP does not function in the late series of Ca2+-triggered reactions leading to membrane fusion but at an early stage of the exocytotic pathway, possibly in the preparation of the docking/fusion machinery.

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