Distinct Effects of α-SNAP, 14-3-3 Proteins, and Calmodulin on Priming and Triggering of Regulated Exocytosis

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Abstract. We have used stage-specific assays for MgATP-dependent priming and for Ca^{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^{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^{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^{2+}-triggered steps immediately prior to membrane fusion as previously suggested.

THE use of cell permeabilization techniques and whole cell patch-clamp recording has yielded much information on the mechanisms and requirements for regulated exocytosis (Burgoyne and Morgan, 1993, 1995). Many questions remain, however, about the sequence of events that lead to exocytosis and the exact roles of proteins involved in this process. In many cell types including adrenal chromaffin cells, exocytosis is activated by micromolar Ca^{2+} and is optimal when millimolar MgATP is provided (Baker and Knight, 1978; Dunn and Holz, 1983; Wilson and Kirschner, 1983). MgATP-dependent secretion requires ATP hydrolysis since non-hydrolysable ATP analogues do not support secretion (Holz et al., 1989; Knight and Baker, 1982).

Regulated exocytosis from permeabilized cells requires cytosolic proteins (Sarafian et al., 1987; Martin and Walent, 1989; Koffer and Gomperts, 1989). Several proteins have now been identified which stimulate exocytosis from permeabilized cells following their addition after cytosolic protein leakage, including annexin II (Ali et al., 1989), 14-3-3 proteins (Morgan and Burgoyne, 1992*a*; Roth et al., 1994; Wu et al., 1992), calmodulin (Okabe et al., 1992), p145 (Walent et al., 1992), phosphatidylinositol transfer protein (Hay and Martin, 1993), phosphatidylinositol-4-phosphate 5-kinase (Hay et al., 1995), protein kinase C (Morgan and Burgoyne, 1992*b*; Nishizaki et al., 1992), protein kinase A (Morgan et al., 1993), and α-SNAP (Morgan and Burgoyne, 1995). Some of these proteins may act as regulators of Ca^{2+}-dependent exocytosis while others may have more fundamental roles in secretory vesicle docking and fusion. In the majority of cases their site and mechanism of action remains to be determined.

Kinetic analysis of exocytosis in neuroendocrine cells has shown that Ca^{2+} activates multiple steps, with different Ca^{2+} affinities, leading to exocytosis (Bittner and Holz, 1992; Neher and Zucker, 1992; Thomas et al., 1993). In addition, in chromaffin and PC12 cells, exocytosis can be functionally dissected into sequential MgATP-dependent (priming) and MgATP-independent (triggering) stages (Bittner and Holz, 1992; Hay and Martin, 1992; Holz et al., 1989) which are stimulated by distinct cytosolic factors (Hay and Martin, 1992). It has been shown that MgATP-dependent priming can allow exocytotic fusion to occur in response to Ca^{2+} in the absence of free MgATP. A lack of an obligate requirement for MgATP in exocytosis has also been demonstrated in *Paramecium* (Vilmart-Seuwen et al., 1986) and complete degranulation can occur in permeabilized mast cells in the nominal absence of free MgATP (Howell et al., 1989). In all of these cell types the primed state is labile and is lost following prolonged incubation without MgATP.

It has been suggested that α-SNAP and NSF function as general components of the fusion machinery in vesicular transport and a model for their actions in neurotransmitter release has been proposed (Söllner et al., 1993*a*,*b*). According to this model synaptic vesicles dock at the plasma membrane via a core docking complex consisting of the vesicle protein synaptobrevin and the plasma membrane

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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. (1993*a*).

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 digitonin-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 digitonin 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 5mM EGTA (pH 6.5). KGEP/MgATP buffer also contained 2 mM ATP and 2 mM $MgCl_2$. Permeabilization buffer was KGEP with 2 mM ATP, 2 mM $MgCl_2$, and 20 μM digitonin added. The published protocol for the purification of Exo1 (Morgan and Burgoyne, 1992*a*) 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)[1] 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 medullae 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, 5mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_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 fluorodeoxyuridine, 50 μg/ml gentamycin, 10 μM cytosine arabinofuranoside, 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 KGEP/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 μl 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 KGEP/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 KGEP/MgATP buffer and various Ca^{2+} concentrations (0–10 μM) for 15 min. Purified proteins, cytosol, or dialysis buffer were included in this step as indicated. Step 3. Incubation with KGEP containing 10 μM Ca^{2+} for 4 min and assay of released catecholamine.

Triggering Assay. Step 1. Incubation with permeabilization buffer for 25 min. Step 2. Incubation with KGEP 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 Floding, 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 ^{32}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 EDTA/HCl/methanol (2:1), vortexed, centrifuged briefly and the upper aqueous phase removed carefully. 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%)/dH_2O (90:90:7:20) as a mobile phase in a saturated chamber. Radioactive phospholipids were visualized with a Molecular Dynamics

1. *Abbreviations used in this paper*: FPLC, fast protein liquid chromotography; NSF, N-ethylmaleimide-sensitive fusion protein; NTA, nitrolotriacetic acid; PtdINS(4,5)P_2, phosphatidylinositol (4,5) bisphosphate; SNAP-25, synaptosomal associated protein of 25 kD; SNAPs, soluble NSF attachment proteins; SNAREs, SNAP receptors; t.l.c., thin layer chromatography.

PhosphorImager (Eugene, OR) and phosphatidylinositol(4,5)bisphosphate identified using unlabelled standard, that had been visualized with molybdenum blue (Sigma).

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 Ca^{2+}-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 Ca^{2+} 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 Ca^{2+} 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) Ca^{2+} concentrations (Bittner and Holz, 1992). In contrast, cytosolic priming factors identified from studies on PC12 cells did not require elevated Ca^{2+} for activity (Hay and Martin, 1992, 1993). In our initial experiments it was found that brain cytosol stimulated priming at 0 Ca^{2+} and additionally showed a Ca^{2+}-dependent effect maximal at 1 μM Ca^{2+} (data not shown). It is probable, therefore, that cytosol contains multiple priming factors that differ in their requirement for Ca^{2+}.

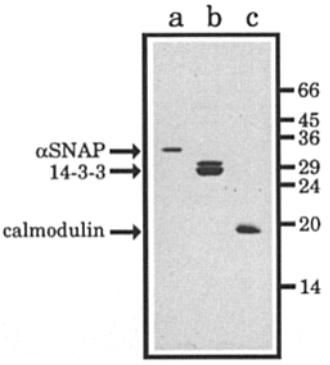

Figure 1. SDS-PAGE analysis of purified proteins used in this study. Coomassie blue stained 12.5% SDS–polyacrylamide gel of recombinant α-SNAP (lane *a*), purified sheep brain 14-3-3 proteins (lane *b*), and purified bovine brain calmodulin (lane *c*).

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 Ca^{2+} in the presence of MgATP (the combined assay since both priming and triggering can occur) each of the proteins increased secretion in a Ca^{2+}-dependent manner (Fig. 2) as expected from the previous work on α-SNAP (Morgan and Burgoyne, 1995), 14-3-3 proteins (Morgan and Burgoyne, 1992*a*) 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 Ca^{2+} concentration of 10 μM whereas that due to 14-3-3 proteins and calmodulin was seen at lower Ca^{2+} 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 Ca^{2+} challenge in the absence of MgATP after incubation with the proteins at various Ca^{2+} 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 Ca^{2+} whereas the stimulatory effect of 14-3-3 pro-

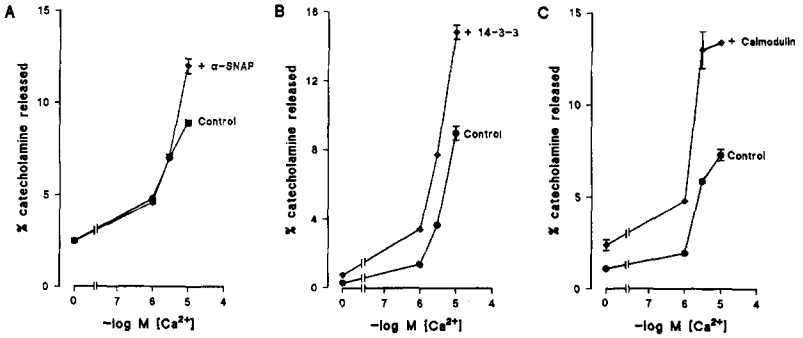

Figure 2. Stimulatory effects of α-SNAP, 14-3-3 proteins, and calmodulin in a combined assay for catecholamine secretion from permeabilized chromaffin cells. Cells were permeabilized for 45 min and then incubated for 15 min in 200 μl per well KGEP/MgATP buffer with the indicated free Ca^{2+} concentration in the presence or absence of 25 μg/ml α-SNAP (*A*), 250 μg/ml 14-3-3 protein (*B*), or 250 μg/ml calmodulin (*C*). Data shown are means ± S.E. (n = 4) and are expressed as percentage of total cellular catecholamine.

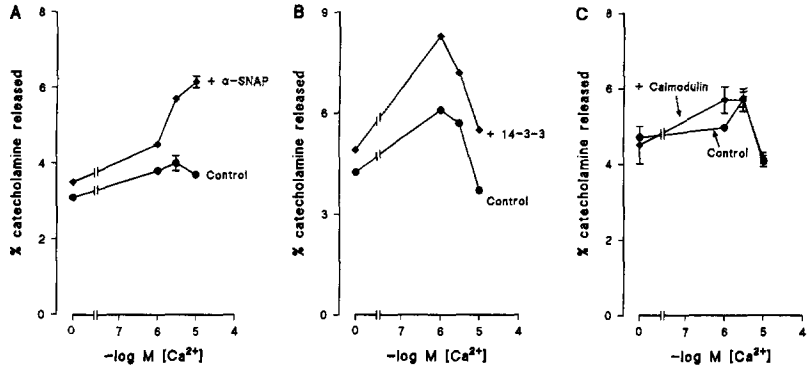

Figure 3. Effects of α-SNAP, 14-3-3 proteins and calmodulin on priming of Ca^{2+}-activated secretion from permeabilized chromaffin cells. Cells were permeabilized for 45 min. The cells were then incubated for 15 min in 200 μl KGEP/MgATP buffer with the indicated free Ca^{2+} concentration in the presence 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.

teins over the control reaction was most marked at 1 μM Ca^{2+} but was detectable at all Ca^{2+} concentrations. Differences between control data in Fig. 3 reflect variations between cell batches but the effects of the added proteins were reproducibly similar in all experiments with at least three separate batches of cells.

If α-SNAP and 14-3-3 proteins acted on different aspects of the priming reaction, as suggested by their different Ca^{2+} dependencies, then their effect should be at least additive if both are present in the priming assay. To test this, a priming assay was carried out with combinations of proteins in the priming step at the optimal Ca^{2+} concentration (10 μM) for priming by α-SNAP. The effects of α-SNAP and 14-3-3 proteins were clearly additive (Fig. 4),

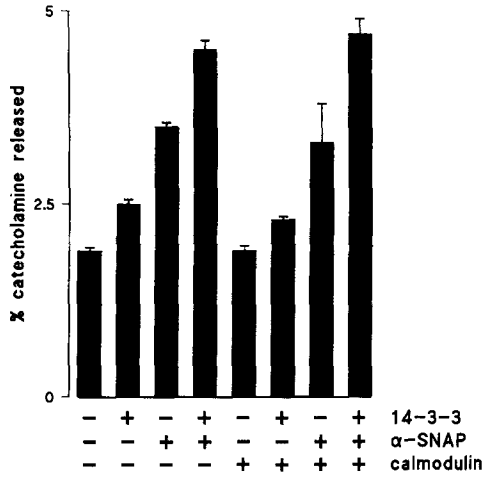

Figure 4. Effects of combinations of α-SNAP, 14-3-3 proteins and calmodulin in the priming reaction. Chromaffin cells were permeabilized for 45 min and incubated for 15 min in 200 μl per well KGEP/MgATP containing 10 μM Ca^{2+} with or without α-SNAP (25μg/ml), 14-3-3 proteins (250 μg/ml), or calmodulin (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.

despite being present at maximal concentrations, consistent with the suggestion that they have distinct mechanisms of action. Calmodulin alone or in combination with α-SNAP or 14-3-3 proteins had no effect on priming. A difference between α-SNAP and 14-3-3 proteins was also observed in experiments in which α-SNAP or 14-3-3 proteins were included with 10 μM Ca^{2+} in the presence of MgATP and the time course of secretion in this combined assay determined. The stimulation by 14-3-3 proteins was complete within 10 min of incubation but α-SNAP continued to increase secretion over control levels for up to 40 min and gave a considerably greater stimulation of secretion than did 14-3-3 proteins (Fig. 5). These data are again consistent with different actions of the two proteins in priming.

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,

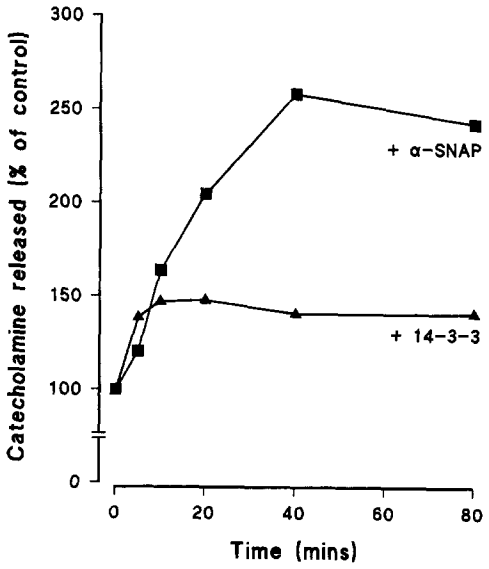

Figure 5. Effect of α-SNAP and 14-3-3 proteins on the time course of Ca^{2+}-activated secretion from permeabilized chromaffin cells in a combined assay. Cells were permeabilized for 45 min and incubated in 300 μl per well KGEP/MgATP containing 10 μM Ca^{2+} without additions (control) or with 25 μg/ml α-SNAP or 250 μg/ml 14-3-3 proteins. Catecholamine release over the indicated times was determined and the amount of catecholamine released in the presence of α-SNAP or 14-3-3 proteins was expressed as a percentage of the control value for each time point (n = 4).

1992, 1993; Hay et al., 1995). Two of these factors appear to act sequentially to enhance synthesis of phosphatidylinositol (4,5) bisphosphate (PtdIns[4,5]P_2) and it has been suggested that PtdIns(4,5)P_2 itself may play a direct role in exocytosis (Hay et al., 1995). We therefore examined the effect of α-SNAP and 14-3-3 proteins on PtdIns(4,5)P_2 synthesis during the priming reaction. Rat brain cytosol was included as a control in these experiments since it should contain the previously described priming factors (Hay et al., 1995) that lead to increased PtdIns(4,5)P_2 synthesis. The presence of cytosol did indeed result in increased PtdIns(4,5)P_2 synthesis (Fig. 7) while incubation in the presence of 10 μM Ca^{2+} reduced the extent of label incorporated presumably due to Ca^{2+}-dependent activation of phospholipase C. Neither α-SNAP nor 14-3-3 proteins had any effect on PtdIns(4,5)P_2 synthesis in the presence or absence of 10 μM Ca^{2+} (Fig. 7).

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, 1992*a*; 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

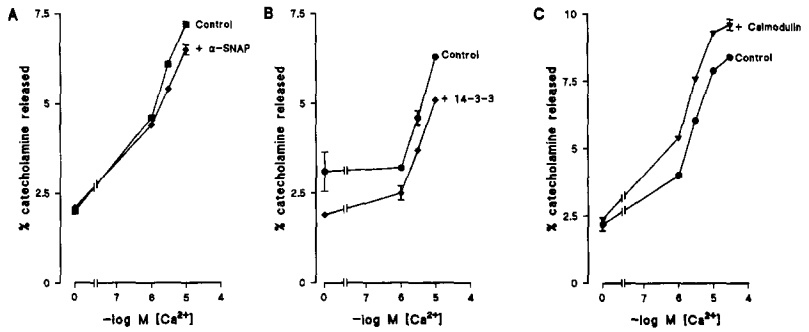

Figure 6. Effect of α-SNAP, 14-3-3 proteins and calmodulin on MgATP-independent Ca^{2+}-activated secretion in a triggering assay. Cells were permeabilized for 25 min and then incubated for 15 min in KGEP containing the indicated free Ca^{2+} concentration with or without 25 μg/ml α-SNAP (*A*), 250 μg/ml 14-3-3 proteins (*B*), or 250 μg/ml calmodulin (*C*). Data shown are means ± S.E. (n = 4) and are expressed as percentage of total cellular catecholamine.

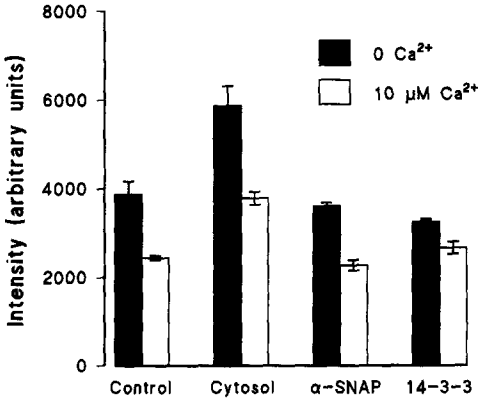

Figure 7. Effect of cytosol, α-SNAP, and 14-3-3 proteins on synthesis of PtdIns(4,5)P_2 during a priming reaction in permeabilized chromaffin cells. Cells were permeabilized for 45 min and then incubated in KGEP/MgATP containing ^{32}P-ATP and with 0 or 10 μM Ca^{2+} with no additions (*control*), 1mg/ml rat brain cytosol, 25 μg/ml α-SNAP, or 250 μg/ml 14-3-3 proteins for 15 min. The extent of synthesis of PtdIns(4,5)P_2 during this priming incubation was determined by t.l.c. analysis of label incorporated into PtdIns (4,5)P_2 and quantified using a Phosphorimager.

several different ATP-dependent steps. Previous work has suggested that priming may involve polyphosphoinositide synthesis (Eberhard and Holz, 1990; Eberhard et al., 1991) and priming factors identified in PC12 cells are proteins in sequential steps in PtdIns(4,5)P_2 synthesis (Hay and Martin, 1993; Hay et al., 1995). Reorganization of the cortical actin cytoskeleton has been implicated in increasing secretory vesicle availability (Burgoyne and Morgan, 1993; Trifaro and Vitale, 1993) and can occur in response to protein kinase C activation (Burgoyne et al., 1989; Vitale et al., 1995) and thus has an MgATP requirement. An additional ATP-dependent step may be mediated by the ATPase NSF (Söllner et al., 1993*a*,*b*). The distinct time courses of action of α-SNAP and 14-3-3 proteins would be consistent with 14-3-3 proteins activating a process which must occur only once for increased secretion, whereas, α-SNAP may be needed continuously during secretion to facilitate multiple rounds of vesicle recruitment, docking and fusion.

There is no evidence that α-SNAP or 14-3-3 proteins are themselves ATP-binding proteins. No effect of these proteins on PtdIns(4,5)P_2 synthesis in permeabilised cells was detected during the priming reaction and so this particular biochemical process cannot underlie the ability of these proteins to stimulate in the priming stage of exocytosis. The function of α-SNAP in the secretory pathway is believed to be in recruitment of NSF to membranes and one model has been suggested for neurotransmitter release in which ATP hydrolysis by NSF after its binding to α-SNAP on the SNARE complex of docked synaptic vesicles is a late event immediately preceding membrane fusion (Söllner et al, 1993a). In the present and in a previous study (Morgan and Burgoyne, 1995) exogenous α-SNAP stimulated exocytosis without the need for addition of exogenous NSF. Similarly, injection of α-SNAP was also shown to produce a slow and long-lasting increase in neurotransmitter release from the squid giant synapse (DeBello et al., 1995). Nevertheless, α-SNAP appears to stimulate in the ATP-dependent priming stage of exocytosis. An important question is whether α-SNAP was acting on exocytosis in these situations in conjunction with NSF. In permeabilized chromaffin cells all treatments that affected the equilibrium of α-SNAP between bound and soluble forms affected NSF in parallel suggesting tight association between these two proteins (Morgan and Burgoyne, 1995). In addition, a proportion of α-SNAP and NSF remained cell associated even after long permeabilization times. It has been demonstrated that α-SNAP does not function solely to bind NSF to the SNARE complex but also activates the ATPase activity of NSF and does so maximally with the stoichiometry in vitro of 2 α-SNAPs per NSF monomer, i.e., 6 α-SNAPs per NSF trimer (Morgan et al., 1994). The data from permeabilized cells can be reconciled as follows. NSF already membrane associated via α-SNAP could be further activated to hydrolyse ATP by binding of additional exogenous α-SNAP. This would explain the ability of α-SNAP to stimulate exocytosis in an ATP requiring step. The data are not consistent, however, with the original model (Söllner et al., 1993*a*) in which ATP hydrolysis by NSF is a late event, but suggest instead that α-SNAP (with NSF) acts in the earlier priming stage to prepare aspects of the exocytotic machinery. α-SNAP and NSF interact with the SNARE proteins (Söllner et al., 1993*a*,*b*) and it may be that hydrolysis of ATP by NSF provides energy for the modification of one or more SNAREs during priming. Indeed, ATP-dependent priming in chromaffin cells results in modification of the toxin sensitivity of synaptobrevin and SNAP-25 (Bittner and Holz, 1993; Lawrence et al., 1994) to which α-SNAP and NSF bind in vitro in extracts of brain (Söllner et al., 1993*a*,*b*) and adrenal medullary (Roth and Burgoyne, 1994) membranes. The change in toxin sensitivity could result if priming lead to an increase in the number of docked vesicles. This would be consistent with recent data showing that α-SNAP binds tightly to the SNARE complex but not to individual SNARE proteins (McMahon and Sudhof, 1995) and so α-SNAP could stimulate vesicle docking. It should be noted, however, that all three SNARE proteins have been detected on synaptic vesicles (Walch-Solimena et al., 1995). Alternatively, therefore, the role of α-SNAP and NSF may be to prime the components of the docking/fusion machinery and this could occur before vesicle docking at the plasma membrane since NSF is present on undocked synaptic vesicles (Hong et al., 1994).

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 (Söllner et al, 1993*a*), which may be a Ca^{2+} 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 (Söllner et al., 1993*a*) and suggests that α-SNAP does not function in the late series of Ca^{2+}-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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