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A role for soluble NSF attachment proteins (SNAPs) in regulated exocytosis in adrenal chromaffin cells

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Digitonin-permeabilized chromaffin cells secrete catecholamines by exocytosis in response to micromolar Ca^{2+} concentrations, but lose the ability to secrete in response to Ca^{2+} as the cells lose soluble proteins through the plasma membrane pores. Such secretory run-down can be retarded by cytosolic fractions, thus providing an assay for proteins potentially involved in the exocytotic process. We have used this assay to investigate the role of *N*-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF attachment proteins (SNAPs) in regulated exocytosis. Recombinant α- and γ-SNAP stimulated Ca^{2+}-dependent exocytosis, although recombinant NSF was ineffective, despite the fact that NSF and α-SNAP leak from the permeabilized cells with similar time courses. However, around one third of cellular NSF was found to be present in a non-cytosolic form and so it is possible that this is sufficient for exocytosis and that exogenous SNAPs stimulate the exocytotic mechanism by acting on the leakage-insensitive NSF. The stimulatory effect of α-SNAP displayed a biphasic dose–response curve and was maximal at 20 μg/ml. The effect of α-SNAP was Ca^{2+}- and MgATP-dependent and was inhibited by *N*-ethylmaleimide and botulinum A neurotoxin, indicating a *bona fide* action on the exocytotic mechanism. Furthermore, Ca^{2+} concentrations which trigger catecholamine secretion acted to prevent the leakage of NSF and α-SNAP from permeabilized cells. These findings provide functional evidence for a role of SNAPs in regulated exocytosis in chromaffin cells.

Key words: calcium/exocytosis/NSF/secretion/SNAPs

Introduction

Ca^{2+} has long been recognized as the major signal leading to exocytosis in many cell types, including adrenal chromaffin cells (Burgoyne, 1991). Although the nature of the Ca^{2+} signal responsible for exocytotic secretion in the chromaffin cell is well understood, the proteins which Ca^{2+} acts upon to bring about the exocytotic fusion of granule and plasma membranes have remained elusive. Recent work has, however, identified a number of candidate proteins (Burgoyne and Morgan, 1993). In permeabilized chromaffin cells (i.e. cells whose plasma membranes have been selectively porated) exocytosis can be directly triggered simply by raising Ca^{2+} concentrations to micromolar levels in the presence of MgATP (Baker and Knight, 1978). However, one drawback of certain permeabilizing agents, such as digitonin and streptolysin O, is that the lesions created in the plasma membrane are so large as to allow the exit (and entry) of cytosolic proteins. This leakage of cytosolic proteins results in a run-down of the ability of the cell to secrete in response to Ca^{2+}. Ironically, this apparent drawback has great potential for identifying those proteins involved in the exocytotic process. It has been shown by Sarafian *et al.* (1987) that re-introduction of the proteins which leak from digitonin-permeabilized chromaffin cells can reconstitute secretion from such run-down cells, indicating that cytosolic proteins are essential for exocytosis. Following this work, a requirement for cytosolic proteins in regulated exocytosis was demonstrated in GH3 cells (Martin and Walent, 1989), PC12 cells (Lomneth *et al.*, 1991), mast cells (Koffer and Gomperts, 1989) and brain synaptosomes (Kish and Ueda, 1991). By using the run-down/reconstitution approach, several soluble proteins (cytosolic and extrinsic membrane proteins) have been identified which regulate exocytosis in neuroendocrine cells, including annexin II (Ali *et al.*, 1989; Ali and Burgoyne, 1990; Sarafian *et al.*, 1991), 14-3-3 proteins (Exo1; Morgan and Burgoyne, 1992a,b; Wu *et al.*, 1992), p145 (Nishizaki *et al.*, 1992; Walent *et al.*, 1992), phosphatidylinositol transfer protein (Hay and Martin, 1993), protein kinase C (Naor *et al.*, 1989; Morgan and Burgoyne, 1992b; Nishizaki *et al.*, 1992) and protein kinase A (Morgan *et al.*, 1993). In these neuroendocrine cells exocytosis is inhibited by the clostridial neurotoxins. These toxins are now known to be zinc proteases (Schiavo *et al.*, 1992a,b) which selectively cleave either synaptobrevin, syntaxin or SNAP-25 (see Huttner, 1993, for review), thus providing functional evidence for an essential role of these three proteins in neurosecretion.

Using analogous reconstitution approaches, Rothman and co-workers have identified several soluble mammalian proteins required for intra-Golgi transport *in vitro* (reviewed in Rothman and Orci, 1992), including *N*-ethylmaleimide-sensitive fusion protein (NSF; Block *et al.*, 1988) and the soluble NSF attachment proteins (SNAPs; Clary and Rothman, 1990; Clary *et al.*, 1990). NSF was subsequently found to be required for *in vitro* transport from the endoplasmic reticulum to the Golgi stack (Beckers *et al.*, 1989) and for endocytic vesicle fusion *in vitro* (Diaz *et al.*, 1989), suggesting that NSF was required for multiple stages of vesicular transport in the constitutive secretory and endocytic pathways. The finding that NSF and α-SNAP are the functional homologues of the yeast *SEC18* (Wilson *et al.*, 1989) and *SEC17* (Clary *et al.*, 1990; Griff *et al.*, 1992) gene products respectively demonstrated a role for these proteins in membrane traffic *in vivo*. NSF and SNAPs were known to assemble into 20S

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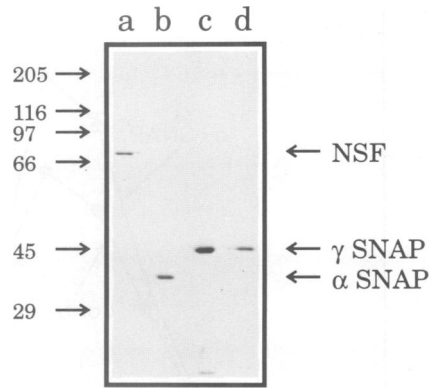

Fig. 1. SDS–PAGE analysis of recombinant His_6-tagged protein samples. Coomassie blue stained 10% SDS–polyacrylamide gel of recombinant proteins used in this study. (a) NSF (0.4 μg); (b) α-SNAP (0.4 μg); (c) γ-SNAP (1 μg); (d) Mono Q (Tris) purified γ-SNAP (0.4 μg).

particles in the presence of detergent-solubilized membranes (Wilson *et al.*, 1992), indicating the presence of membrane-bound SNAP receptors (SNAREs). Using an immunoprecipitation approach with brain membranes, these SNAREs were identified as synaptobrevin, syntaxin and SNAP-25 (Sollner *et al.*, 1993a); the three proteolytic substrates for the clostridial neurotoxins (Huttner, 1993). This discovery had tremendous impact, since it suggested that the same soluble proteins (NSF and SNAPs) active in constitutive secretion were also involved in the tightly regulated process of neurotransmitter release. However, although this idea has rapidly become accepted, it is based solely on the indirect approach of *in vitro* binding of proteins to detergent extracts (Sollner *et al.*, 1993a,b). In the present paper we have attempted to redress this situation by testing the ability of recombinant NSF and SNAPs to stimulate regulated exocytosis in digitonin-permeabilized chromaffin cells. Chromaffin cells are ideal for such a study, since they are one of the few non-neuronal cell types which are known to express syntaxin, synaptobrevin (Hodel *et al.*, 1994) and SNAP-25 (Roth and Burgoyne, 1994) and are sensitive to clostridial neurotoxins (Bittner *et al.*, 1989). Here we report functional evidence for a role of SNAPs in regulated exocytosis.

Results

Expression of recombinant proteins and purification on nickel–nitrilotriacetic acid–agarose (Ni–NTA–agarose) yielded large quantities of essentially pure NSF and α-SNAP as judged by SDS–PAGE (Figure 1). However, γ-SNAP required a further purification step in order to achieve a comparable level of purity (Figure 1). In order to assess a possible role of these proteins in regulated exocytosis they were used in experiments on digitonin-permeabilized chromaffin cells. NSF was purified on the basis of its ability to reconstitute intra-Golgi transport which had been blocked by treatment with *N*-ethylmaleimide (NEM; Block *et al.*, 1988). In order to see whether such treatment could directly reveal an NSF requirement in regulated exocytosis, chromaffin cells were pre-treated with NEM using a similar protocol [1 mM NEM on ice for 15 min, followed by a 2 mM dithiothreitol (DTT)

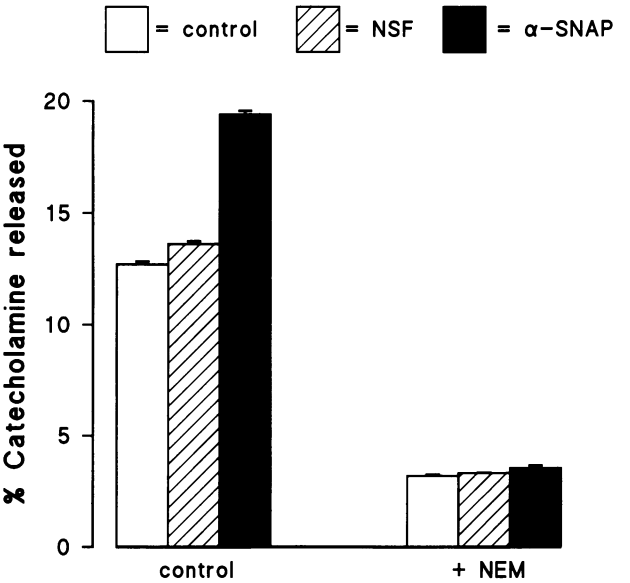

Fig. 2. Effect of NSF and α-SNAP on exocytosis in control and NEM-treated cells. Cells were pretreated on ice with ice-cold Ca^{2+}-free Krebs–Ringer buffer in the presence or absence of 1 mM NEM for 15 min, then incubated for a further 5 min in ice-cold Ca^{2+}-free Krebs–Ringer buffer in the presence (for NEM-treated cells) or absence of 2 mM DTT. Cells were then returned to room temperature and permeabilized for 75 min with buffer A containing 20 μM digitonin and an ATP regenerating system (10 mM phosphocreatine, 8 U/ml creatine phosphokinase) in the presence of 29 μg/ml α-SNAP, 30 μg/ml NSF or buffer for controls, prior to stimulation in buffer A containing 10 μM Ca^{2+}. Data shown are means ± SE (n = 4) and are expressed as a percentage of total cellular catecholamine.

quench on ice for 5 min]. NEM treatment abolished Ca^{2+}-dependent secretion, but this inhibition could not be reversed by NSF or α-SNAP in either acute (data not shown) or prolonged (Figure 2) incubations with the permeabilized cells or even by brain cytosol (data not shown). These experiments did reveal, however, that secretion in control cells was stimulated by α-SNAP. In experiments with proteins alone or in combination (Figure 3) it was seen that either α- or γ-SNAP stimulated Ca^{2+}-dependent secretion and that the effect of the two combined was less than additive. γ-SNAP was stimulatory in the presence of a supra-maximal dose of α-SNAP (200 μg/ml), indicating that α- and γ-SNAP function independently in stimulating exocytosis, as they do in intra-Golgi transport (Whiteheart *et al.*, 1993). In contrast, NSF from two separate batches had little effect on catecholamine release in the presence or absence of recombinant SNAPs. This lack of a stimulatory effect of NSF was not due to inactivation of the protein during the purification process, as NSF ATPase activity was detected in the same samples (data not shown). Using the standard protocol, α-SNAP alone enhanced Ca^{2+}-dependent exocytosis in all 21 experiments performed, γ-SNAP alone enhanced secretion in three of the eight experiments performed, while no significant stimulatory activity was found for NSF in any of the 10 experiments performed using NSF concentrations ranging from 0.1 to 200 μg/ml. Studies were also performed using NSF which had been further purified by gel filtration chromatography on Superdex 200, but this material was also unable to stimulate Ca^{2+}-dependent exocytosis.

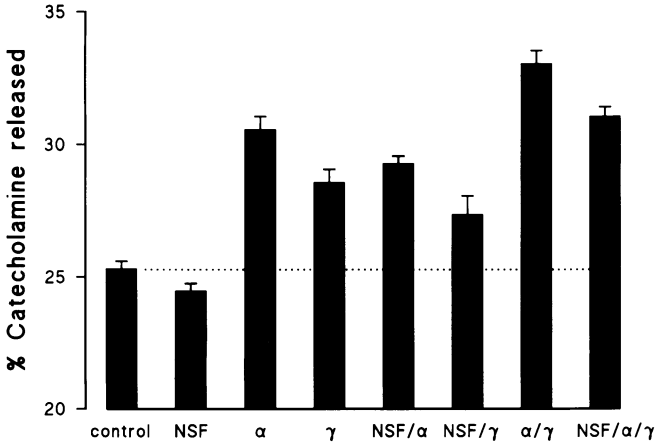

Fig. 3. Effect of NSF, α- and γ-SNAP on Ca^{2+}-dependent exocytosis in permeabilized cells. Cells were permeabilized for 15 min in buffer A containing 20 μM digitonin and then incubated for a further 30 min with buffer A containing 1 mM DTT and 0.02% NaN_3 and 200 μg/ml NSF, 200 μg/ml α-SNAP or 4 μg/ml γ-SNAP (alone or in combination) prior to stimulation in buffer A containing 10 μM Ca^{2+}. Data shown are means ± SE (n = 4) and are expressed as a percentage of total cellular catecholamine. The dotted line indicates the control level of release for comparison.

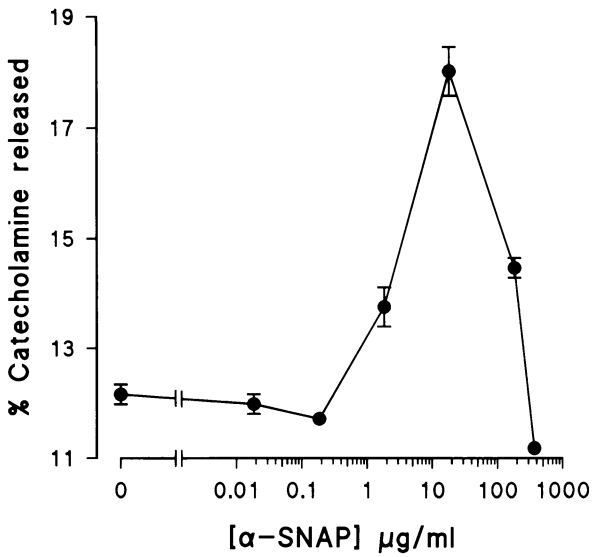

Fig. 4. Dose dependency of the stimulatory effect of α-SNAP. Cells were permeabilized for 15 min in buffer A containing 20 μM digitonin and then incubated for a further 30 min with buffer A containing 1 mM DTT and 0.02% NaN_3 and the indicated concentrations of α-SNAP prior to stimulation in buffer A containing 10 μM Ca^{2+}. Data shown are means ± SE (n = 4) and are expressed as a percentage of total cellular catecholamine. In some cases the SE was smaller than the symbols.

Since the stimulation by α-SNAP was more consistent, the effects of SNAPs were characterized using α-SNAP. The stimulatory effect of α-SNAP displayed a biphasic dose–response curve which was half-maximal at 3.5 μg/ml, maximal at 20 μg/ml and had returned to control levels by 300 μg/ml (Figure 4). Catecholamine release is triggered by micromolar Ca^{2+} in permeabilized chromaffin cells (Baker and Knight, 1978) and so the Ca^{2+} dependency of the α-SNAP stimulation was investigated. It was found that α-SNAP had little effect up to 300 nM Ca^{2+}, but enhanced release in response to higher concentrations,

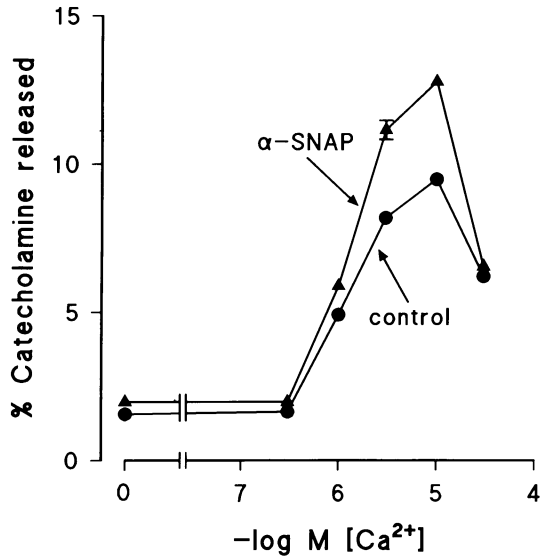

Fig. 5. Ca^{2+} dependency of the stimulatory effect of α-SNAP. Cells were permeabilized for 15 min in buffer A containing 20 μM digitonin and then incubated for a further 30 min with buffer A containing 1 mM DTT and 0.02% NaN_3 in the presence or absence of 18.5 μg/ml α-SNAP prior to stimulation in buffer A containing the indicated free Ca^{2+} concentrations. Data shown are means ± SE (n = 4) and are expressed as a percentage of total cellular catecholamine. In some cases the SE was smaller than the symbols.

with maximal stimulation occurring at 10 μM Ca^{2+}; the optimum for control secretion (Figure 5). By analogy with the constitutive secretory and endocytic pathways (Rothman and Orci, 1992), it is likely that α-SNAP stimulates regulated exocytosis by acting on endogenous NSF and that α-SNAP levels are limiting under the conditions used here. NSF is an ATPase (Tagaya *et al.*, 1993; Whiteheart *et al.*, 1994) and ATP hydrolysis is essential for its action (Sollner *et al.*, 1993b; Whiteheart *et al.*, 1994). If α-SNAP stimulates chromaffin cell exocytosis by acting in concert with NSF, then its action should be entirely MgATP-dependent. Indeed, significant α-SNAP stimulation was only apparent in the continuous presence of MgATP; omission of MgATP from stage 2, i.e. incubation with α-SNAP, or stage 3, i.e. stimulation with Ca^{2+}, attenuated the stimulation by α-SNAP (Figure 6).

Recently, NSF and SNAPs have been found to physically interact in a complex with the synaptic proteins syntaxin, synaptobrevin and SNAP-25 (Sollner *et al.*, 1993a). These proteins are all proteolytic substrates for clostridial neurotoxins and so it has been suggested that NSF/SNAPs elicit neurotransmitter release via this interaction (Sollner *et al.*, 1993a,b). In order to test this hypothesis directly, the ability of α-SNAP to stimulate exocytosis was examined in the presence of a modified form of botulinum A neurotoxin (BoNT/A LH_N; Shone *et al.*, 1987). Botulinum A neurotoxin partially inhibits exocytosis in digitonin-permeabilized chromaffin cells (Bittner *et al.*, 1989) and the toxin is now known to specifically cleave SNAP-25 (Blasi *et al.*, 1993; Schiavo *et al.*, 1993). BoNT/A LH_N (100 nM) inhibited control Ca^{2+}-dependent exocytosis by only 22%, but inhibited the α-SNAP stimulation by 68% (Figure 7), suggesting that α-SNAP exerts its effect on the exocytotic process by a SNAP-25-dependent mechan-

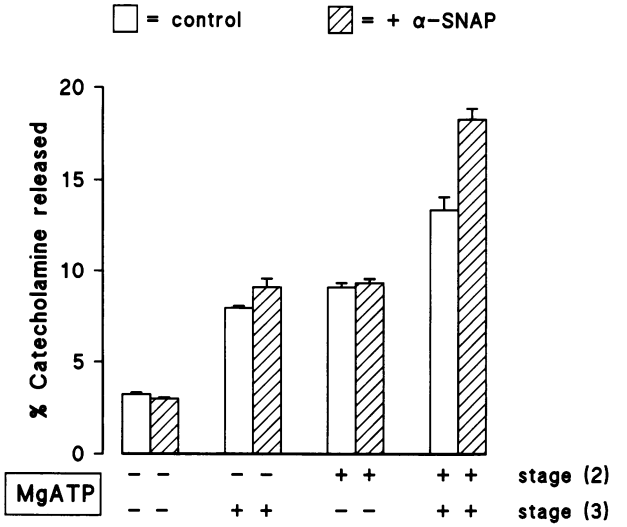

Fig. 6. The stimulatory effect of α-SNAP is MgATP-dependent. Cells were permeabilized for 10 min in buffer A containing 20 μM digitonin in stage 1 in the presence of 2 mM MgATP as in the standard protocol. In stage 2, cells were incubated for 15 min with 30 μg/ml α-SNAP, or dialysis buffer for controls, in the presence (+) or absence (−) of 2 mM MgATP. In stage 3, cells were stimulated to secrete by challenge with 10 μM Ca^{2+} in the presence (+) or absence (−) of 2 mM MgATP. Data shown are means ± SE (n = 6) and are expressed as a percentage of total cellular catecholamine.

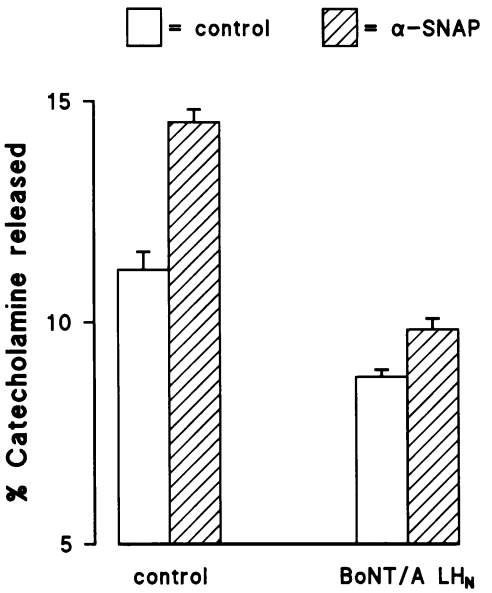

Fig. 7. The stimulatory effect of α-SNAP is botulinum neurotoxin A-sensitive. Cells were permeabilized for 10 min in buffer A containing 20 μM digitonin and then incubated for a further 15 min with buffer A containing 1 mM DTT and 0.02% NaN_3 in the presence or absence of 100 nM BoNT/A LH_N and 29 μg/ml α-SNAP (alone or in combination) prior to stimulation in buffer A containing 10 μM Ca^{2+}. Basal (Ca^{2+}-independent) release was 1.32 ± 0.04 and 1.52 ± 0.04% in the presence and absence of BoNT/A LH_N respectively and these values were subtracted from the values obtained in the presence of 10 μM Ca^{2+} to yield the Ca^{2+}-dependent release values shown. Data shown are means ± SE (n = 4) and are expressed as a percentage of total cellular catecholamine.

ism. In order to determine whether the stimulatory effect of α-SNAP required other soluble proteins, permeabilized cells were allowed to run down for progressively longer

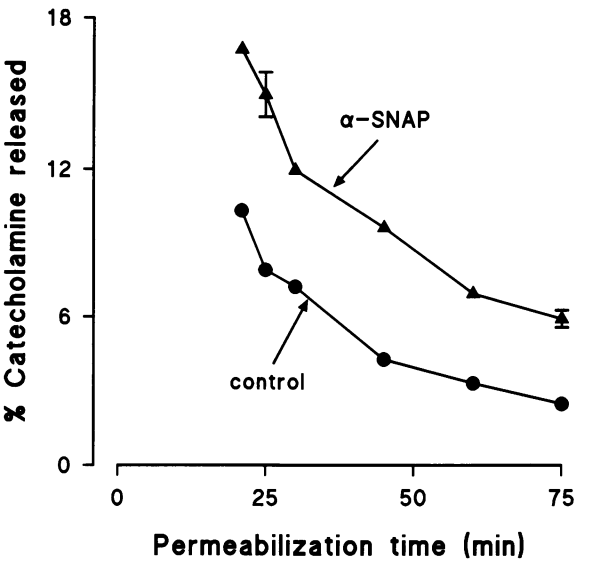

Fig. 8. α-SNAP stimulates exocytosis in extensively run-down cells. Cells were permeabilized in buffer A containing 20 μM digitonin for varying times and then incubated for a further 15 min with buffer A containing 1 mM DTT and 0.02% NaN_3 and 29 μg/ml α-SNAP prior to stimulation in buffer A containing 10 μM Ca^{2+}. Permeabilization time indicates the time period prior to stimulation, i.e. stages 1 and 2 combined. Data shown are means ± SE (n = 4) and are expressed as a percentage of total cellular catecholamine. In some cases the SE was smaller than the symbols.

periods prior to α-SNAP incubation (Figure 8). It can be seen that the stimulatory effect of α-SNAP was roughly constant irrespective of the amount of run-down, suggesting that α-SNAP acts on a component which either does not leak from the cells or is so large that its leakage is not rate-limiting. NSF is an attractive candidate for such a component (see below).

In using the run-down/reconstitution approach to test for a functional role of a given protein, two assumptions are made: first, that the protein is expressed in chromaffin cells; second, that the protein leaks from the permeabilized cells. In order to test these assumptions, chromaffin cells were permeabilized for progressively longer periods and the proteins in the supernatant and those retained in the cells examined by immunoblotting using antisera to NSF and α-SNAP. Figure 9 shows that both proteins are present in chromaffin cells and both leak from the cells upon permeabilization. Interestingly, despite the large difference in molecular mass between NSF (228 kDa in trimeric form; Whiteheart *et al.*, 1994) and α-SNAP (33 kDa; Whiteheart *et al.*, 1993), the time course of leakage/retention is similar for both, with maximal leakage occurring within 40 min (Figure 9). Furthermore, there is a proportion of NSF and α-SNAP (around one third and one half respectively) which does not leak from the cells. It is possible that this non-cytosolic pool of NSF represents the run-down-resistant component which exogenous α-SNAP acts on to stimulate catecholamine secretion (Figure 8). Since regulated exocytosis in chromaffin cells requires Ca^{2+} (Figure 5) and MgATP (Figure 6) and is inhibited by NEM (Figure 3), the effects of these treatments on leakage/retention of NSF and α-SNAP were investigated and are shown in Figure 10. It can be seen that incubation without MgATP or pre-treatment of the cells with NEM

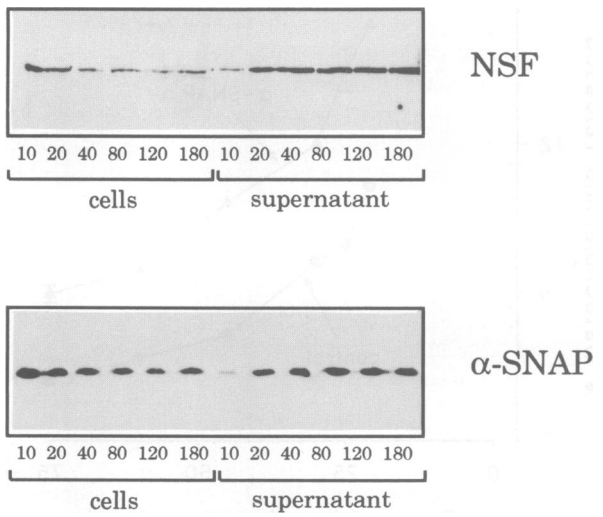

Fig. 9. Time-dependent leakage of NSF and α-SNAP from permeabilized chromaffin cells. Cells were incubated for 10, 20, 40, 80, 120 and 180 min in buffer A containing 20 μM digitonin. After the indicated times, the supernatants were centrifuged to remove cell debris and then both supernatants and cells were precipitated with −20°C methanol before solubilization in 300 μl SDS sample buffer and boiling. Samples were run on 10% SDS−polyacrylamide gels and Western blotted overnight. Immunoblotting was performed using anti-NSF at 1:100 and anti-α-SNAP at 1:3000. After 180 min of permeabilization, 32.1% of NSF was cell-associated and 67.9% had leaked from the cells, while 44.7% of α-SNAP was cell-associated and 55.3% had leaked from the cells.

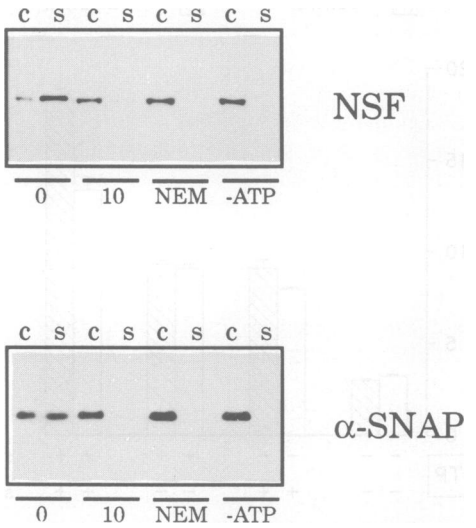

Fig. 10. Ca^{2+}, NEM and lack of MgATP prevent leakage of both NSF and α-SNAP from permeabilized chromaffin cells. Cells were incubated on ice for 15 min in Ca^{2+}-free Krebs−Ringer buffer in the presence or absence of 1 mM NEM. Cells were then incubated on ice for a further 5 min in Ca^{2+}-free Krebs−Ringer buffer in the presence (for NEM-treated cells) or absence of 2 mM DTT. Cells were then removed from ice and incubated for 60 min in buffer A containing 20 μM digitonin (0, NEM), buffer A containing 20 μM digitonin and 10 μM free Ca^{2+} (10) or buffer A containing 20 μM digitonin but lacking MgATP (−ATP). After 60 min, the supernatants were centrifuged to remove cell debris and then both supernatants and cells were precipitated with −20°C methanol before solubilization in 300 μl SDS sample buffer and boiling. Samples were run on 10% SDS−polyacrylamide gels and Western blotted overnight. Immunoblotting was performed using anti-NSF at 1:100 and anti-α-SNAP at 1:3000. Under control conditions (0), 40% of NSF was cell-associated and 60% had leaked from the cells, while 50.4% of α-SNAP was cell-associated and 49.6% had leaked from the cells. After challenge with Ca^{2+} (10), 86.8% of NSF was cell-associated and 13.2% had leaked from the cells, while 81.3% of α-SNAP was cell-associated and 18.7% had leaked from the cells. In the NEM and −ATP treatments, essentially all of the NSF and α-SNAP was cell-associated.

totally prevented the leakage of both NSF and α-SNAP and that 10 μM Ca^{2+} greatly reduced their leakage. These effects appeared specific, as there was no comparable change in the overall protein staining of the blots and since immunoblotting with antisera to 14-3-3 protein (γ isoform) revealed little differences between treatments (data not shown). The NEM-induced retention of (inactivated) NSF in the permeabilized cells may explain the inability of NSF, or indeed cytosol, to reconstitute exocytosis from such NEM-treated cells. Moreover, the observation that micromolar Ca^{2+} can influence the cellular localization of NSF and α-SNAP provides evidence for a link between the trigger for regulated exocytosis and NSF/SNAPs and is consistent with Ca^{2+} driving the assembly of a multi-subunit fusion machine containing NSF and SNAPs.

Discussion

The work of Rothman and colleagues has firmly established an essential role for NSF and SNAPs in the constitutive secretory and endocytic pathways (Rothman and Orci, 1992). These proteins were identified on the basis of their ability to reconstitute vesicular transport *in vitro*. Following the spectacular success of this approach, reconstitution studies were applied to regulated exocytotic secretion, resulting in the identification of a number of different candidate proteins (reviewed in Burgoyne and Morgan, 1993). Sollner *et al.* (1993b) have proposed an explicit model in which SNAPs and NSF bind to a docking complex (SNAREs) of neurotoxin-sensitive vesicle and plasma membrane proteins and the subsequent hydrolysis of ATP by NSF provides the driving force for membrane fusion. We have found that both α- and γ-SNAP stimulate Ca^{2+}-regulated release of catecholamine from digitonin-permeabilized chromaffin cells. The action of α-SNAP is Ca^{2+}- and MgATP-dependent and is inhibited by NEM and botulinum A neurotoxin, suggesting that it exerts its stimulatory effect via the exocytotic process. Maximal stimulation with α-SNAP occurred at 20 μg/ml. This is higher than the amount required for maximal activity in intra-Golgi transport assays (around 2 μg/ml; Whiteheart *et al.*, 1993), although this is probably due to the restricted diffusion of proteins through the plasma membrane pores in digitonin-permeabilized cells.

The effects of SNAPs in vesicular transport appear to be entirely mediated by interactions with NSF and so the stimulatory activity of SNAPs seen in this study can be assumed to reflect a requirement for NSF in regulated exocytosis in chromaffin cells. Nevertheless, stimulatory effects of recombinant NSF were not observed here, despite the fact that α-SNAP and NSF leak from permeabilized chromaffin cells with similar time courses; raising the possibility that SNAPs function independently of NSF in exocytosis. This paradox can be resolved if it is assumed that NSF is expressed at supra-maximal levels

in chromaffin cells and that SNAP expression is limiting. If so, the non-cytosolic pool of NSF (around one third of total NSF) may be sufficient for a full exocytotic response and exogenous SNAPs may act on this leakage-insensitive NSF to stimulate Ca^{2+}-dependent exocytosis. This theory is supported by the observation that stimulatory effects of His_6-NSF on endocytic vesicle fusion cannot be demonstrated unless the membranes are first washed with high salt to remove endogenous membrane-associated NSF (Rodriguez *et al.*, 1994). In an attempt to directly demonstrate stimulation by NSF in chromaffin cells, the NEM treatment protocol used originally to identify NSF (Block *et al.*, 1988) was applied. Such treatment abolished regulated exocytosis, indicating an essential role for an NEM-sensitive protein (NSF?), but neither NSF nor brain cytosol could reverse its inhibition. This result is similar to that seen in transferrin recycling *in vitro*, which is inhibited by anti-NSF antibodies but cannot be reconstituted by cytosol following inactivation with NEM (Podbilewicz and Mellman, 1990). Thus, it may be that another NEM-sensitive protein(s) is required for exocytosis, as has been suggested for endocytic vesicle fusion (Rodriguez *et al.*, 1994). Alternatively, as such NEM treatment was found to prevent the leakage of NSF from permeabilized chromaffin cells, it may be that NEM-inactivated NSF remaining in the cell interferes with the exocytotic response. The finding that Ca^{2+} concentrations which elicit catecholamine secretion act to prevent the leakage of α-SNAP and NSF from permeabilized cells indicates a link between the trigger for catecholamine release and NSF/SNAPs, implying a functional role for both proteins in regulated exocytosis.

Recently, NSF and SNAPs have been shown to bind in a 20S complex with synaptobrevin, syntaxin and SNAP-25 (Sollner *et al.*, 1993a). The latter three proteins are known to be essential for neurotransmitter release *in vivo*, as they are proteolytic substrates for clostridial neurotoxins (Huttner, 1993), suggesting that NSF/SNAPs mediate neurotransmission by physically interacting with these proteins to form a multi-subunit fusion machine. A similar complex containing NSF, synaptobrevin, syntaxin, SNAP-25 (Roth and Burgoyne, 1994) and α-SNAP (D.Roth, personal communication) has recently been isolated from adrenal medullary membranes, suggesting that the same set of proteins mediate regulated exocytosis in both neurons and chromaffin cells. The demonstration here that α-SNAP stimulates Ca^{2+}-dependent catecholamine release supports this hypothesis and thus represents the first functional evidence for a role for SNAPs in regulated exocytosis. Further support for this hypothesis comes from the observation here that the α-SNAP stimulation is inhibited by botulinum A neurotoxin, which specifically proteolyzes SNAP-25 (Blasi *et al.*, 1993; Schiavo *et al.*, 1993), indicating that the mechanism by which α-SNAP triggers exocytosis involves SNAP-25. Further work by Sollner *et al.* (1993b) has shown that α-SNAP and the Ca^{2+} binding protein synaptotagmin compete for the same binding site in the SNARE complex, leading to the suggestion that synaptotagmin acts as a fusion clamp which, upon binding Ca^{2+}, allows α-SNAP and NSF to bind and elicit ATP-driven membrane fusion. It should be pointed out, however, that Ca^{2+} had no effect on the abundance of synaptotagmin in the SNARE complex *in vitro* (Sollner *et al.*, 1993b). The observation here that physiological Ca^{2+} concentrations prevent the leakage of both α-SNAP and NSF from permeabilized cells is consistent with Ca^{2+} driving the assembly of a multi-subunit fusion machine containing NSF and SNAPs. The role of synaptotagmin in this process remains undetermined, although this protein is present in the SNARE complex in adrenal medulla (Roth and Burgoyne, 1994).

The basic tenet of the Sollner *et al.* (1993a,b) model is that a v-SNARE must bind to its cognate t-SNARE match before α-SNAP, and subsequently NSF, can bind to form a 20S particle. In the present study, we found that around one third of cellular NSF and one half of cellular α-SNAP exist in a non-cytosolic form, despite the presence of MgATP. Interestingly, treatments known to inactivate NSF (NEM, ATP depletion) prevented the leakage of not only NSF but also α-SNAP, implying that the two proteins are complexed in some way prior to secretory stimuli. In chromaffin cells, around 99% of secretory granules are kept at least 200 nm away from the plasma membrane by a dense network of cytoskeletal actin which is only disassembled in response to secretagogues (Burgoyne and Morgan, 1993). Clearly, this forbids docking of the v-SNARE (synaptobrevin) on the granule with t-SNAREs (syntaxin, SNAP-25) on the plasma membrane under resting conditions and, indeed, docked granules are rarely seen in these cells. Since α-SNAP and NSF are retained in the cell in the absence of a Ca^{2+} challenge, this must mean that NSF/SNAPs are able to form complexes with either v- or t-SNAREs alone or, alternatively, that NSF/SNAPs are bound to membranes via different (i.e. non-SNARE-dependent) interactions. These findings, coupled with the observation that NSF is bound to synaptic vesicles in the absence of Ca^{2+} influx (Hong *et al.*, 1994), indicate that the precise sequence of the protein−protein interactions proposed in the Sollner *et al.* (1993b) model may require revision.

The stimulatory effect of α-SNAP was observed in extensively run-down chromaffin cells, indicating that this protein acts on a component which does not leak from the cells. Since around one third of cellular NSF is leakage-insensitive, it seems likely that α-SNAP stimulates exocytosis by acting on this non-cytosolic pool of NSF. This effect of α-SNAP contrasts with the stimulatory activity of annexin II, which is inactive after such a prolonged run-down period (Burgoyne and Morgan, 1990). It appears then that in permeabilized chromaffin cells, a portion of the Ca^{2+}-dependent response, in which annexin II is involved, requires soluble proteins and is thus leakage-sensitive. A further portion, which is stimulated by α-SNAP, is leakage-insensitive, since the magnitude of the α-SNAP stimulation was not reduced by increasing permeabilization time. One possible explanation for this difference is if annexin II and α-SNAP act on distinct parallel pathways in the exocytotic mechanism. Indeed, separate mechanisms of action of annexin II and NSF/SNAPs have been suggested for endosome−endosome fusion (reviewed in Burgoyne and Clague, 1994). An alternative explanation is provided by observations that Ca^{2+}-dependent exocytosis can be divided into multiple stages which can be discriminated on the basis of kinetics, ATP dependency and Ca^{2+} dependency (see Morgan and Burgoyne, 1993, for a review). Thus, it may be that

annexin II and α-SNAP act on distinct stages of the exocytotic mechanism which have different requirements for cytosolic proteins. If so, since α-SNAP still stimulates exocytosis after the leakage of soluble proteins required by annexin II, it seems likely that α-SNAP acts on a stage beyond that involving annexin II in the exocytotic pathway.

In conclusion, the results presented here provide strong functional evidence for a role for SNAPs in regulated exocytosis. As the effects of SNAPs in vesicular transport appear to be entirely mediated by interactions with NSF, this further suggests a requirement for NSF in regulated exocytosis, and this is supported by a variety of evidence presented here. Nevertheless, since exogenous NSF was unable to stimulate Ca^{2+}-dependent secretion, it is formally possible that SNAPs function independently of NSF in exocytosis. Specific reagents which can inactivate the non-cytosolic endogenous NSF will be required to address this point. A major future undertaking will be to determine whether the soluble proteins previously shown to stimulate regulated exocytosis in permeabilized cells, such as annexin II, 14-3-3 proteins, p145 and phosphatidylinositol transfer protein, interact with the NSF–SNAP–SNARE complex, act on different stages of the same exocytotic mechanism or trigger exocytosis by a separate, parallel pathway. The cell permeabilization approach described here will be invaluable to such studies and the use of stage-specific permeabilized cell assays (Holz *et al.*, 1989; Hay and Martin, 1993) and patch-clamp capacitance techniques (Neher and Zucker, 1993; Thomas *et al.*, 1993) should allow the function of these proteins, including NSF/SNAPs, to be assigned to the various steps in regulated exocytosis.

Materials and methods

Materials

Plasmids encoding His_6-tagged NSF, α-SNAP and γ-SNAP were generously donated by Dr J.E.Rothman (Memorial Sloan-Kettering Cancer Center, New York, NY). Anti-α-SNAP antiserum raised against recombinant His_6-α-SNAP was a gift from Dr T.Levine (ICRF Laboratories, London, UK). The botulinum A neurotoxin fragment LH_N was prepared as described previously (Shone *et al.*, 1987) and was a gift from Dr C.C.Shone (Centre for Applied Microbiology and Research, Salisbury, UK). *Escherichia coli* XL-1 Blue were obtained from Stratagene (Cambridge, UK). Nickel–nitrilotriacetic acid–agarose (Ni–NTA–agarose) was obtained from Hybaid (Middlesex, UK). 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).

Expression and purification of recombinant proteins

This was based on a previously published method using plasmids for NSF, α- and γ-SNAP and purification of His_6-tagged proteins on Ni–NTA–agarose (Whiteheart *et al.*, 1993). The poor expression of γ-SNAP resulted in a heterogenous pattern on SDS–PAGE relative to recombinant NSF and α-SNAP (Figure 1) and so γ-SNAP which had been further purified on Mono Q in Tris buffer as previously described (Clary and Rothman, 1990) was used in some experiments to ensure that the effects were not due to contaminating bacterial proteins. All chromatography was performed at 4°C using a Pharmacia FPLC system. Protein concentrations were estimated using the method of Bradford (1976). SDS–PAGE was performed using a Bio-Rad mini Protean II according to the manufacturer's instructions.

Preparation of affinity-purified anti-NSF antibodies

His_6-NSF (300 μg) in complete Freund's adjuvant was injected subcutaneously at multiple sites into a rabbit, followed after 4 weeks by a similar injection in incomplete Freund's adjuvant. Bleeds were taken at 6 weeks and thereafter at 2–4 week intervals. Each bleed was accompanied by boosting with 300 μg His_6-NSF without adjuvant. Affinity purification was performed using 1 mg His_6-NSF linked to a 0.5 ml Ni–NTA–agarose column. Serum was incubated batchwise with the column for 1 h at room temperature with agitation and then the column was washed with Ni–NTA–agarose loading buffer [50 mM imidazole, 200 mM KCl, 2 mM β-mercaptoethanol, 0.5 mM ATP, 10% glycerol (v/v), 20 mM HEPES, pH 7] to remove non-specifically bound proteins. Antibodies were eluted with 3 ml 5 M LiCl in 10 mM KH_2PO_4, at pH 7.2, and dialysed against buffer A (139 mM potassium glutamate, 2 mM ATP, 2 mM $MgCl_2$, 5 mM EGTA, 20 mM PIPES, pH 6.5) before storage at −20°C. Immunoblotting was performed using the method of Roth and Burgoyne (1994). The antibody did not cross-react with His_6-α-SNAP and recognized a single band of around 70 kDa in homogenates of rat forebrain and bovine adrenal medulla, thus demonstrating its specificity.

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 Ca^{2+}-free Krebs–Ringer buffer, consisting of 145 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose and 20 mM HEPES, 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 1×10^6 cells/well and maintained in culture for 3–7 days before use.

Cell permeabilization and assay of catecholamine secretion

After initial washing of cells in Ca^{2+}-free Krebs–Ringer buffer, the standard protocol was in three stages: (1) Cells were permeabilized with 300 μl buffer A containing 20 μM digitonin for 10 or 15 min; (2) cells were incubated with 200 μl recombinant proteins in buffer A containing 1 mM DTT and 0.02% NaN_3 for 15 min or longer, as indicated in the legends; and (3) Cells were stimulated with 300 μl buffer A containing 10 μM free Ca^{2+} for 20 min. After stage 3, the buffer was removed, centrifuged at 16 000 *g* for 2 min and aliquots taken for assay of released catecholamine.

Assay of released catecholamines was performed using a fluorimetric 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 total cellular catecholamine. All experiments were performed at room temperature. Basal catecholamine release, i.e. release in response to challenge with buffer A without added Ca^{2+} in stage 3, was not altered by the treatments used unless indicated otherwise and was consistently low; in 46 experiments from 36 different cell preparations, basal catecholamine release was 1.61 ± 0.09%.

α-SNAP and γ-SNAP fractions were dialyzed overnight against buffer A containing 1 mM DTT and 0.02% NaN_3 before use in secretion experiments. NSF was stored in Ni–NTA–agarose elution buffer [250 mM imidazole, 200 mM KCl, 2 mM β-mercaptoethanol, 0.5 mM ATP, 10% glycerol (v/v), 20 mM HEPES, pH 7] and diluted into buffer A containing 1 mM DTT and 0.02% NaN_3 for secretion experiments, using elution buffer as a control.

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