Similar effects of α- and β-SNAP on Ca^{2+}-regulated exocytosis

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Abstract Soluble *N*-ethylmaleimide-sensitive fusion protein attachment proteins (SNAP) proteins function in Ca^{2+}-regulated exocytosis. Recent work (Schiavo et al. (1996) *Nature* 378, 733–736) based on in vitro protein interactions has raised the possibility that α- and β-SNAPs have distinct roles in exocytosis. We have examined this possibility by comparing the activities of recombinant α- and β-SNAPs. Both of these proteins were able to similarly bind NSF and activate its ATPase activity but to a lesser extent than γ-SNAP. When introduced into digitonin-permeabilised chromaffin cells, both α- and β-SNAP stimulated Ca^{2+}-regulated exocytosis in a MgATP-dependent manner. The dose-response relationships for these proteins were essentially the same and addition of both proteins did not lead to any further increase in exocytosis above that due to each protein alone. We conclude that α- and β-SNAPs are interchangeable isoforms with similar functions in regulated exocytosis.

Key words: Exocytosis; Calcium; Secretion; SNAP; Chromaffin cell

1. Introduction

Key proteins that act in Ca^{2+}-regulated exocytosis in neurons and endocrine cells include the vesicle proteins synaptotagmin [1] and VAMP/synaptobrevin [2,3], the plasma membrane proteins syntaxin [4] and SNAP-25 [5] and, in addition, the soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) [16] and soluble NSF-attachment proteins (α-, β- and γ-SNAPs) [7] Functional evidence for the importance of the membrane proteins has come from their sensitivity to the specific proteolytic actions of clostridial neurotoxins [8,9] and/or genetic analysis in mice and *Drosophila* [10–12]. The soluble factors NSF and SNAP were found to interact, in a 20S complex, with the neurotoxin substrates leading to them being designated as SNAP-receptors (SNARE) [13,14]. Functional evidence that NSF functions in regulated exocytosis comes from the *comatose* mutant in *Drosophila* in which a temperature-sensitive defect in NSF leads to a block in neurotransmission [15]. Exogenous α-SNAP stimulates exocytosis after injection into the squid giant synapse [16] or introduction into adrenal chromaffin cells after digitonin permeabilisation [17–19] or during patch-clamp recording [20] supporting a role for this protein in Ca^{2+}-regulated exocytosis.

NSF and SNAPs were originally detected as factors required for transport through the Golgi in in vitro assays [6,7,21] and the yeast homologues of these proteins, sec18 and sec17, are essential for secretion in vivo [22]. In Golgi transport assays and in the formation of a Golgi membrane derived 20S complex, α- and β-SNAP appear to be functionally redundant whereas γ-SNAP binds to different membrane sites and has a distinct role in stabilisation of the 20S complex [23,24]. Since γ-SNAP supports transport poorly [21] and is not essential for in vitro Golgi transport its exact physiological role is unclear. β-SNAP is expressed mainly in brain and since α- and β-SNAP have 83% sequence identity [21] one possibility is that they are simply functionally similar isoforms. This view is supported by the finding of a single SNAP in *Drosophila* [25] and also squid [16] with sequences, in each case, which are equally related to those of both mammalian α- and β-SNAPs. Consistent with its apparently distinct function, γ-SNAP shows only 25% identity to α- and β-SNAPs.

In contrast to earlier analysis of α- and β-SNAP function in Golgi transport, more recent work has suggested the possibility that α- and β-SNAPs may have distinct functions in regulated exocytosis based on the apparent ability of β- but not α-SNAP to bind to the putative Ca^{2+} sensor synaptotagmin I [26] In addition, the affinity of binding of β-SNAP to a synaptic SNARE complex was found to be 2- to 3-fold lower than that of α-SNAP. We have tested the possibility that α- and β-SNAP function in different aspects of regulated exocytosis using permeabilised chromaffin cells. These provide an ideal system for such a study since exogenous α-SNAP stimulates Ca^{2+}-regulated exocytosis in chromaffin cells [17–20] and synaptotagmin I, which is the isoform demonstrated to bind β-SNAP [26] is the major synaptotagmin in these cells [27]. The functional data presented here suggests that α- and β-SNAP have interchangeable roles in regulated exocytosis.

2. Materials and methods

2.1. Materials

Restriction endonucleases, *Taq* polymerase, T4 DNA ligase, reverse transcription system, and plasmid DNA purification kits were all obtain from Promega (Southampton, UK). RNeasy RNA isolation kit, pQE-30 plasmid DNA, Ni^{2+}–NTA agarose, and *E. coli* M15[pREP4] cells were obtained from Qiagen (Surrey, UK). DNA primers were obtained from Cruachem (Glasgow, UK), high purity digitonin was obtained from Novabiochem (Nottingham, UK) and all other chemicals were of an analytical grade from Sigma (Poole, UK). Plasmids encoding His_6-NSF, His_6–α-SNAP and His_6–γ-SNAP were gifts from Dr. J.E. Rothman (Memorial Sloan Kettering Cancer Centre, New York).

2.2. Plasmid production

Total RNA was extracted from whole rat brain using an RNeasy isolation kit, cDNA synthesised with a reverse transcription system and cDNA encoding β-SNAP amplified in PCR reactions using an Omn-E dryblock thermocycler (Hybaid, Middlesex, UK). The sense and antisense primers used were (5′-CGGGATCCATGGACAA-

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Abbreviations: Ni^{2+}–NTA, nickel–nitriloacetic acid; NSF, *N*-ethylmalemide-sensitive fusion protein; PCR, polymerase chain reaction; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor

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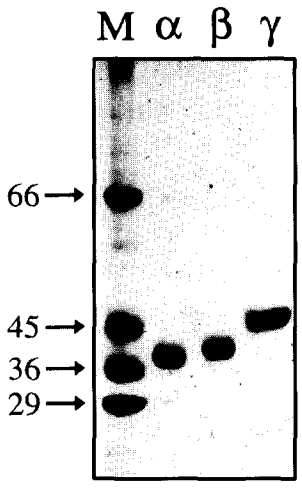

Fig. 1. SDS-PAGE analysis of purified recombinant His_6-tagged SNAPs. The figure shows Coomassie Blue-stained 12.5% SDS-polyacrylamide gel of Ni-NTA-agarose purified α-, β-, and γ-SNAP in comparison to molecular mass standards (M) indicated in kilodaltons.

CTCCGGGAAGG-3′) and (5′-GTCGGTACCTCACTTGAGGTC-TCCATCTC-3′) respectively. The primers contained *Bam*HI (sense) and *Kpn*I (antisense) endonuclease sites (underlined) to allow subcloning into the pQE-30 vector (Qiagen). PCR consisted of an initial denaturation cycle (95°C for 5 min), 30 cycles of annealing (55°C for 1 min), elongation (72°C for 2 min) and denaturation (95°C for 1 min), a final elongation cycle (72°C for 10 min) and cooling (30°C for 1 min). The single PCR product and pQE-30 plasmid were digested overnight at 37°C with *Bam*HI and *Kpn*I, gel-purified using a Wizard PCR DNA purification kit (Promega) and ligated together using T4 DNA ligase according to suppliers protocol (Promega). The ligation mixture was used to transform competent *E. coli* M15[pREP4] cells (Qiagen). From automated sequencing the cDNA insert encoded a protein with an identical amino acid sequence to that of bovine β-SNAP apart from an isoleucine to valine substitution at position 179 (also present in mouse β-SNAP [28]) and an alanine to serine substitution at position 4.

2.3. Expression and purification of recombinant His_6-tagged proteins

Recombinant protein expression was induced with 2 mM isopropyl-1-thio-β-D-galactopyranoside at 37°C for 5 h and His_6-tagged proteins purified from the cytosolic fraction of M15[pREP4] cells on Ni–NTA–agarose based on previously published methods [21] His_6-tagged proteins were eluted from the Ni–NTA–agarose by applying a 50–500 mM imidazole gradient. His_6–NSF was further purified by gel filtration using a Superdex 200 column. All chromatography was performed at 4°C using a Pharmacia FPLC system. Peak fractions containing recombinant proteins were identified by SDS-polyacrylamide electrophoresis, and the pooled stock solutions stored at −70°C until required.

2.4. NSF binding and ATPase assays

Binding of NSF to SNAPs immobilised on polypropylene tubes was carried out as described previously with 2.5–5 μg of SNAP and 2 μg of NSF added per tube [19]. Following washing of the tubes, and solubilisation of bound proteins in SDS-dissociation buffer, bound proteins were analysed by SDS-polyacrylamide gel electrophoresis (PAGE) and quantitative densitometry. ATPase assays were carried out as described previously using 20 μg per tube of added SNAPs and 1 μg per tube of NSF with released inorganic phosphate being measured after incubation at 37°C for 1 h [29].

2.5. Preparation of chromaffin cell cultures and assay of catecholamine secretion

Chromaffin cells were isolated from bovine adrenal medulla by enzymic digestion and maintained in culture as described [30]. For secretion assays [30], the cells were permeabilised in permeabilisation buffer (139 mM potassium glutamate, 20 mM Pipes, 5 mM EGTA, 2 mM ATP, 2 mM $MgCl_2$, pH 6.5) containing 20 μM digitonin for 45 min and then challenged with permeabilisation buffer containing no Ca^{2+} or 10 μM free Ca^{2+} with or without added protein and catecholamine released over a 15 min period assayed. Where indicated ATP and $MgCl_2$ were omitted from the buffer used in the stimulation step. All experiments were performed at room temperature (22–25°C).

3. Results and discussion

For comparative purposes all three mammalian SNAPs (α, β, γ) were prepared as His_6-tagged recombinant proteins (Fig. 1) and their in vitro properties compared. Initial experiments examined the ability of each SNAP to bind to and activate the ATPase activity of His_6-tagged NSF [29] taking advantage of a solid phase assay in which the SNAPs interact with NSF. All three SNAPs stimulated the ATPase activity of NSF when immobilised on polypropylene tubes (Fig. 2) with maximal stimulation at 10 μg/ml added SNAP in each case. The increase in ATPase activity was abolished by prior treatment of the NSF with 1 mM NEM. The extent of stimulation by α- and β-SNAPs (≈2-fold) was essentially the same. The α- and β-SNAPs became immobilised to the tubes to the same extent and bound NSF to the same molar ratio (Fig. 2). In contrast, γ-SNAP consistently activated the ATPase activity of NSF to a greater extent (≈4–5-fold) and this could be explained, at least in part, by a greater molar ratio of NSF bound to immobilised γ-SNAP (Fig. 2). These assays demonstrate that the His_6-tagged α- and β-SNAPs show similar in vitro interactions with NSF but both differ from γ-SNAP in their effectiveness in binding and activating NSF.

The stimulation of Ca^{2+}-regulated exocytosis in chromaffin cells by exogenous α-SNAP has been previously characterised in detail. As shown in Fig. 3, α- and β-SNAPs stimulated catecholamine release in response to 10 μM Ca^{2+} from digi-

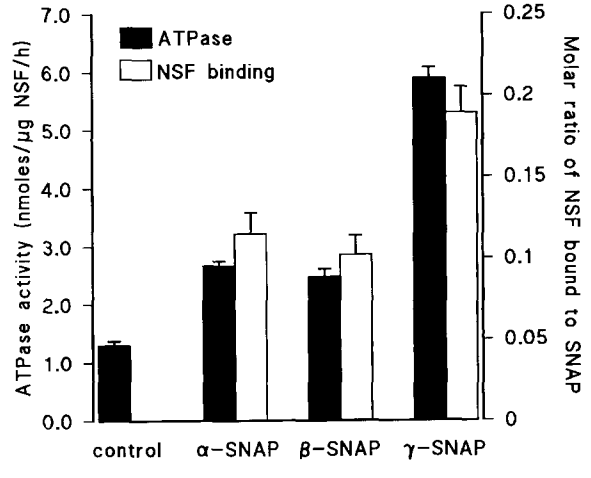

Fig. 2. Binding of His_6-tagged α-, β-, and γ-SNAP to NSF and activation of NSF ATPase activity. SNAPs were immobilised by binding to the surface of polypropylene tubes and after washing NSF was added. Bound proteins were solubilised in SDS-dissociation buffer and analysed by SDS-PAGE and densitometry compared to standards. The ATPase activity of NSF was assayed by measurement of inorganic phosphate release over a 1 h incubation in the absence (control) or presence of maximal concentrations (20 μg added per tube) of each SNAP. γ-SNAP activated the NSF ATPase activity to levels 221 and 238% above that due to α-, β-SNAP, respectively and bound NSF to levels 164 and 168% greater than α- and β-SNAP. For binding, $n = 8$; for ATPase activation $n = 52$ apart from β-SNAP where $n = 12$.

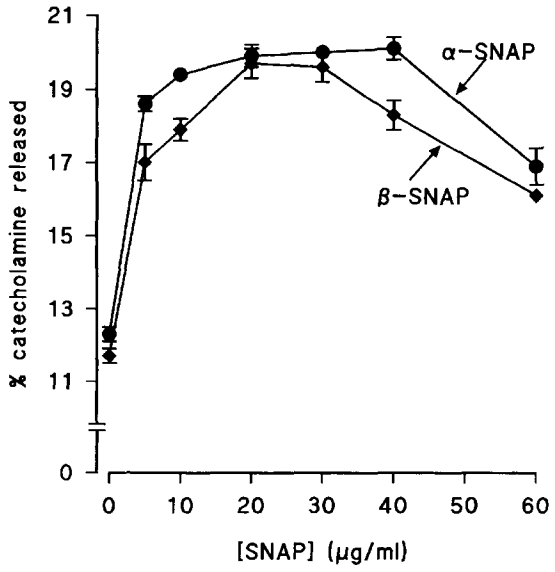

Fig. 3. Dose dependence of the stimulation of Ca^{2+}-dependent catecholamine release from digitonin-permeabilised chromaffin cells by α- and β-SNAPs. Chromaffin cells were permeabilised for 45 min and then stimulated by addition of 10 μM Ca^{2+} in the absence or presence of the indicated concentrations of SNAPs. Catecholamine release over 15 min was assayed and calculated as a percentage of total cellular catecholamine and expressed as mean ± SEM (n = 4-6).

tonin-permeabilised chromaffin cells to the same extent and with essentially identical dose-response relationships. The stimulation of secretion usually declined at higher SNAP concentrations. Neither α- nor β-SNAP had any effect on catecholamine release in the absence of Ca^{2+}. The stimulatory effect of α-SNAP was previously shown to be MgATP-dependent [17,18] and a similar requirement for MgATP was found for β-SNAP stimulation of catecholamine release (Fig. 4). We have previously found that simultaneous addition of exogenous proteins with distinct effects on the exocytotic pathway leads to at least additive effects on catecholamine release [18] and this was the case for combined additions of α- and γ-SNAPs [17]. If α- and β-SNAP have distinct actions due to independent interactions with the syntaxin/SNAP-25/VAMP SNARE complex or synaptotagmin I, respectively, the prediction would be that the effect of the exogenous proteins on catecholamine release would be at least additive and likely synergistic at maximal doses of each protein. In order to test this prediction the effect of α- and β-SNAP alone or in combination at maximal doses was examined. As shown in Fig. 5. The effect of combined addition of both α- and β-SNAP was no different from either SNAP alone consistent with functionally redundant effects of the two proteins. Addition of an even higher concentration of β-SNAP (60 μg/ml) did not result in any further stimulation of catecholamine release above that due to α-SNAP alone.

Previous comparative analysis of the role of α-, β- and γ-SNAPs in intra-Golgi transport suggested that α- and β-SNAP are functionally redundant isoforms but that γ-SNAP has distinct roles [23,24]. This is consistent with the high level of sequence identity between α- and β-SNAP compared to the divergent γ-SNAP. The amino acids that differ between α- and β-SNAP are not conserved in *Drosophila* [15] and squid [16] SNAPs which instead are equally similar to both mammalian SNAPs. Examination of a *C. elegans* SNAP homologue in the Genbank database (accession number U53180) gives a similar picture. It would seem, therefore, that the differences in sequence between α- and β-SNAP would not be predicted to be functionally significant as they occur at non-conserved residues. In one study both α- and β-SNAP were found to associate with the synaptic SNARE complex and it was stated that α- and β-SNAP, but not γ-SNAP, could support NSF-mediated disassembly of the 20S complex in the presence of MgATP [31]. With these indications that α- and β-SNAP are interchangeable isoforms it was surprising, therefore, that it was claimed that they differed in their binding in vitro to GST-synaptotagmin I implying that they could have different roles in regulated exocytosis. It should be noted, however, that no evidence is available to indicate that β-SNAP binds to synaptotagmin I in vivo. The in vitro interaction of β-SNAP with GST-synaptotagmin I allowed recruitment of NSF and this complex was stable in the presence of MgATP [26] in contrast to the 20S complex in which ATP hydrolysis by NSF leads to disassembly [13,14]. One explanation for the stability of the β-SNAP/synaptotagmin/NSF complex could have been that β-SNAP unlike α-SNAP, did not stimulate the ATPase activity of NSF. We have shown here, however, that immobilised β-SNAP, as previously shown for α-SNAP [29], can activate the ATPase activity of NSF in vitro. The significance of the in vitro interactions between β-SNAP and synaptotagmin remains unclear.

α-SNAP stimulates catecholamine release in permeabilised chromaffin cells in a Ca^{2+}- and MgATP-dependent manner [17,18] and this effect is likely to occur via its interactions with SNAREs as it is inhibited by treatment of cells with botulinum A neurotoxin to cleave SNAP-25 [17] and deletion mutants of α-SNAP unable to bind syntaxin do not stimulate exocytosis [19,20]. We have now shown that β-SNAP stimulates Ca^{2+}-regulated catecholamine release with the same dose-dependency and MgATP requirement as α-SNAP. In previous work, the divergent γ-SNAP had small and variable effects on catecholamine release but in some experiments po-

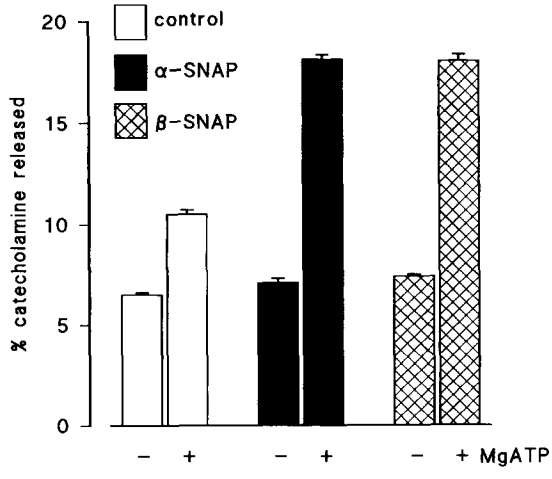

Fig. 4. MgATP-dependency of stimulation of Ca^{2+}-dependent catecholamine release from digitonin-permeabilised chromaffin cells by α- and β-SNAPs. Chromaffin cells were permeabilised for 45 min in the presence of MgATP and then stimulated by addition of 0 or 10 μM Ca^{2+} in the presence or absence of MgATP with or without 25 μg/ml α-SNAP or 20 μg/ml β-SNAP. Ca^{2+}-dependent catecholamine released over 15 min was calculated as a percentage of total cellular catecholamine and expressed as mean ± SEM (n = 4).

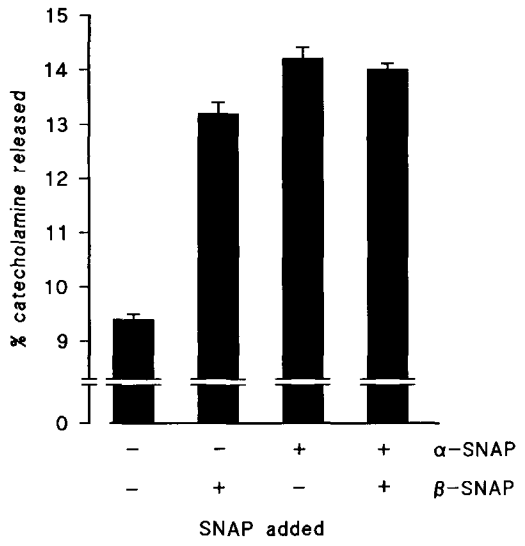

Fig. 5. Non-additivity of α- and β-SNAP stimulation of Ca^{2+}-dependent catecholamine release from digitonin-permeabilised chromaffin cells. After permeabilisation for 45 min the cells were challenged with 10 μM Ca^{2+} in the absence or presence of 25 μg/ml SNAP or 20 μg/ml β-SNAP as indicated and catecholamine release over 15 min assayed ($n = 6$).

tentiated the effect of α-SNAP even when α-SNAP was added to the cells at supra maximal doses [17]. γ-SNAP may, therefore, have a distinct functional role in regulated exocytosis since it apparently does not bind to syntaxin or SNAP-25 [31]. In contrast, no additional effect of a combination of α- and β-SNAP was seen on catecholamine secretion over that due to either of these SNAPs alone. We can not formally rule out that β-SNAP function via synaptotagmin I is accounted for by endogenous β-SNAP. The recent observations of Schiavo et al. [26] have clearly raised the possibility that α- and β-SNAP function in distinct ways in the regulated exocytotic machinery. However, our results do not provide support for distinct functional roles for α- and β-SNAP in Ca^{2+}-regulated exocytosis in chromaffin cells but instead suggest that they are interchangeable isoforms with similar functions.

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