Membrane fusion of secretory vesicles of the sea urchin egg in the absence of NSF

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Summary

The role of cytosolic ATPases such as N-ethylmaleimide (NEM)-sensitive fusion protein (NSF) in membrane fusion is controversial. We examined the physiology and biochemistry of ATP and NSF in the cortical system of the echinoderm egg to determine if NSF is an essential factor in membrane fusion during Ca^{2+}-triggered exocytosis. Neither exocytosis in vitro, nor homotypic cortical vesicle (CV) fusion required soluble proteins or nucleotides, and both occurred in the presence of non-hydrolyzable analogs of ATP. While sensitive to thiol-specific reagents, CV exocytosis is not restored by the addition of cytosolic NSF, and fusion and NSF function are differentially sensitive to thiol-specific agents. To test participation of tightly bound, non-exchangeable NSF in CV-CV fusion, we cloned the sea urchin homolog and developed a species-specific antibody for western blots and physiological analysis. This antibody was without effect on CV exocytosis or homotypic fusion, despite being functionally inhibitory. NSF is detectable in intact cortices, cortices from which CVs had been removed and isolated CVs treated with ATP-γ-S and egg cytosol to reveal NSF binding sites. In contrast, isolated CVs, though all capable of Ca^{2+}-triggered homotypic fusion, contain less than one hexamer of NSF per CV. Thus NSF is not a required component of the CV fusion machinery.

Key words: Sea urchin, Egg, Exocytosis, Fusion, Cortical vesicles

Introduction

Cortical vesicles (CVs) are protein-filled secretory vesicles found in most animal eggs (Peres and Bernardini, 1985; Chandler, 1991; Tahara et al., 1996). At fertilization, CVs undergo exocytosis in response to the elevated cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{free}$) triggered by gamete fusion (Swann and Whitaker, 1986). In sea urchin eggs, the fully primed CVs exist, already docked to the plasma membrane (PM), and disruption of unfertilized eggs in Ca^{2+}-free medium produces sheets of plasma membrane with the CVs firmly attached (Vacquier, 1975; Zimmerberg et al., 1985). In such an egg cortex preparation, exocytosis is triggered by the addition of micromolar $[Ca^{2+}]_{free}$ (Baker and Whitaker, 1978; Sasaki and Epel, 1983; Zimmerberg and Liu, 1988; Blank et al., 1998), and this ionic signal is the only requirement for fusion in vitro (Whitaker, 1987). In addition to fusion with the PM, isolated CVs can also fuse with each other (Vogel and Zimmerberg, 1992) in response to micromolar concentrations of Ca^{2+}, showing that the vesicles themselves possess the entire machinery required for Ca^{2+}-dependent membrane fusion. Characterization of the mechanism of CV-CV fusion has shown that it is the same as for exocytosis in vitro (Coorssen et al., 1998).

A central issue that remains controversial concerns the identity of the protein components of the intracellular membrane fusion machine. Several proteins play important roles in the movement of proteins between the endoplasmic reticulum and other intracellular compartments, pathways that also involve membrane fusion (Rothman, 1994; Lin and Scheller, 2000; Brunger, 2001). These include the cytosolic proteins N-ethylmaleimide (NEM)-sensitive fusion protein (NSF), a hexameric ATPase (Whiteheart et al., 1994; Tagaya et al., 1993; Clary and Rothman, 1990) and soluble NSF-attachment proteins or SNAPs (Clary and Rothman, 1990; Clary et al., 1990); as well as a complex of membrane-bound proteins called SNAP receptors or SNAREs (Wilson et al., 1992; Whiteheart et al., 1992). A heterotrimeric complex of SNARE proteins is reportedly able to catalyze fusion of liposomes (Weber et al., 1998), although evidence from some physiologically relevant biological systems seems to indicate that SNARE protein complexes are not involved in the final stages of membrane fusion in native membranes (Coorssen et al., 1998; Ungermann et al., 1998a; Ungermann et al., 1998b; Peters et al., 2001). Indeed, recent work that has analyzed the sensitivity of SNARE proteins and Ca^{2+}-triggered fusion to a variety of proteases or to blockade by exogenous SNARE binding proteins, suggests that membrane fusion requires additional proteins that function

downstream of SNAREs (Coorssen et al., 2003; Szule et al., 2003).

NSF was proposed originally to form an essential part of the fusion mechanism (Rothman, 1994; Söllner et al., 1993) based on its ubiquitous role in intracellular membrane fusion events. However, experiments in a number of different cell types indicate that ATP hydrolysis by NSF is not required at the precise time of membrane fusion during exocytosis (Baker and Whitaker, 1978; Howell et al., 1987; Holz et al., 1989; Whalley et al., 1991; Hay and Martin, 1992; Parsons et al., 1995) or during other intracellular processes (Mayer et al., 1996; Mayer and Wickner, 1997; Xu et al., 1997). Experiments involving yeast vacuoles show that NSF primes vesicles through unpairing of SNARE complexes residing in the same membrane (cis-SNARE complexes), so that trans-SNARE complexes might form upon contact of vesicle and plasma membrane (Ungermann et al., 1998a; Ungermann et al., 1998b). In addition, NSF may play a role in the recycling of SNAREs, by unpairing cis SNARE complexes that result from fusion (when the two membranes fully merge and all of the SNAREs are resident in the same membrane).

Despite these studies, the role of NSF in intracellular membrane fusion has remained uncertain, due in part to recent reports of lipid vesicle fusion catalyzed by NSF and α-SNAP (Otter-Nilsson et al., 1999; Brugger et al., 2000). In addition, mutants of NSF that lack both ATPase activity and the ability to dissociate SNARE complexes are able to support the fusion of post-mitotic Golgi vesicles into cisternae (Muller et al., 1999). This finding suggests a second potential role for NSF in membrane fusion, beyond a role in priming or recycling of SNARE complex proteins and one that does not require ATP. This once again opens up the possibility that NSF is an integral component of the cellular membrane fusion machinery. Thus the role of NSF in membrane fusion processes is still somewhat unclear.

The work reported here was designed to add clarity to the role of NSF in membrane fusion. We have determined whether NSF plays any direct role in membrane fusion using the exocytosis of secretory vesicles in sea urchin eggs as a model. Sea urchin egg CV exocytosis is sensitive to thiol-specific agents (Haggerty and Jackson, 1983; Vogel et al., 1992; Whalley and Sokoloff, 1994) in common with membrane fusion events in which NSF is thought to participate (Rothman, 1994), although with lower apparent affinity for thiol reagents compared with NSF. In contrast to intracellular fusion events in which NSF is involved (Rothman, 1994), the fusion activity of sea urchin cell surface complexes (CSCs) inhibited with NEM was not restored by the addition of cytosol and Mg-ATP (Whalley and Sokoloff, 1994; Zimmerberg et al., 1996). However, in those experiments only a high concentration (5 mM) of NEM was used to inhibit exocytosis, and it is possible that at this concentration of NEM, alkylation may have occurred at secondary, non-sulfhydryl amino acid residues, which could have resulted in inactivation of proteins other than NSF. Therefore, we assessed the ability of cytosol to restore fusion in CSCs that were partially inhibited with a lower concentration of NEM as well as with more hydrophilic inhibitors of exocytosis, D10PDP and AMSDS, that are likely to react with fewer thiol groups than does NEM (Whalley and Sokoloff, 1994). Sea urchin NSF was cloned and a sensitive species-specific antibody developed. The antibody was functionally inhibitory and this property was used to investigate whether active NSF was a requirement for membrane fusion during exocytosis and homotypic CV membrane fusion.

Materials and Methods

Obtaining and handling eggs

Sea urchins of the species *Strongylocentrotus purpuratus* were purchased from Marinus (Long Beach, CA, USA) or from Westwind Sealab Supplies (Victoria, BC, Canada) and were maintained in aquaria in artificial sea water (435 mM NaCl, 40 mM $MgCl_2$, 15 mM $MgSO_4$, 11 mM $CaCl_2$, 10 mM KCl, 10 mM N-(2-hydroxyethyl) piperazine-N′-2-ethanesulfonic acid (Hepes), 1 mM ethylenediamine-tetraacetic acid (EDTA), pH 8.0) at a temperature of 11°C. Eggs were obtained by injecting 0.5 M KCl into the intracoelomic cavity and the jelly coat was removed by passing through 90 μm nylon mesh. Eggs were kept on ice until use.

Reagents

ATP, adenosine 5′-(3-O-thiotriphosphate) (ATP-γ-S), adenylyl imidodiphosphate (AppNHp), dithiothreitol (DTT) and protease inhibitors were purchased from Boehringer Mannheim (Indianapolis, IN, USA), bovine serum albumin was from ICN (Costa Mesa, CA, USA). Alkaline phosphatase-conjugated sheep anti-rabbit IgG was from Pierce (Rockford, IL, USA) and enhanced chemifluorescence reagent was from Amersham (Pistcataway, NJ, USA). 10 kDa dextran-pyridyldithiopropionate (D10PDP) was prepared as previously described (Whalley and Sokoloff, 1994), 4-acetamido-4′-maleimidylstilbene-2, 2′disulfonic acid (AMSDS) was from Molecular Probes (Eugene, OR, USA). All other reagents were of the highest grade available and were purchased from Sigma Chemical Corp. (St Louis, MO, USA). Monoclonal anti-NSF antibody (#6E6) and a bacterial vector expressing epitope-tagged Chinese hamster NSF were generously provided by Dr James E. Rothman (Sloan-Kettering Memorial Cancer Center, New York, NY). Polyclonal antibody to sea urchin NSF was obtained by injecting rabbits with a multi-antigenic peptide of the sequence SREHFMHAMTDTDIKP-AFGVSSEK$_4$K$_2$K (Covance, Princeton, NJ) and was affinity purified using the same peptide attached to Affigel 15 (BioRad, Hercules, CA). An antibody against hyaline was kindly provided by Dr Gary Wessel (Brown University, Providence, RI, USA).

Preparation of cell surface complexes (CSCs)

CSCs were prepared on ice, essentially as previously described (Whalley and Sokoloff, 1994). Briefly, eggs were washed three times in artificial seawater, three more times in intracellular medium (IM: 220 mM K glutamate, 500 mM glycine, 10 mM NaCl, 5 mM $MgCl_2$, 10 mM ethylene glycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM benzamidine HCl, 2.5 mM MgATP, 5 mM DTT, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 20 μg/ml leupeptin, pH 6.8), and were transferred to a Potter homogenizer. CSC were prepared by homogenization using five or six strokes of a tight-fitting Teflon pestle until a microscopic examination showed that no intact eggs remained. After homogenization, CSCs were pelleted at 700 ***g*** for 1 minute and resuspended in fresh IM. Centrifugation was repeated until the preparation appeared free of contamination and consisted only of large sheets of plasma membrane and associated CVs, as assessed in a phase contrast microscope.

Measurement of exocytosis

Exocytosis was measured essentially as described previously (Whalley and Sokoloff, 1994). Briefly, CSCs were suspended in IM

to give an absorbance of between 0.4 and 0.6 at 405 nm. 100 μl aliquots were dispensed into flat-bottomed microtiter plates (Costar, Cambridge, MA, USA). Exocytosis was triggered by the addition of 100 μl of Ca^{2+}-containing IM to give the required $[Ca^{2+}]_{free}$, which was verified with the use of a Ca^{2+}-selective electrode (World Precision Instruments, Sarasota, FL, USA). Exocytosis was determined by measuring A_{405} in duplicate using a ThermoMax microtiter-plate reader (Molecular Devices, Menlo Park, CA). The extent of exocytosis was normalized by taking A_{405} in 0.1 μM Ca^{2+}-containing IM as 0% exocytosis and A_{405} in 1 mM Ca^{2+}-containing IM as 100% exocytosis.

Preparation of isolated planar cortices (IPCs)

Washed and de-jellied eggs were allowed to adhere as a confluent monolayer to 9 cm diameter plastic Petri dishes treated with 200 μg/ml polylysine. The adherent eggs were washed three times with IM and lysed by squirting with a stream of IM. The IPCs were washed twice in IM, twice in PKME (50 mM 1,4-piperazine-diethanesulfonic acid (Pipes), 425 mM KCl, 10 mM $MgCl_2$, 5 mM EGTA, 1 mM benzamidine HCl, 5 mM DTT, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 20 μg/ml leupeptin, pH 6.8) for a total of 6 minutes in order to deplete adhering endoplasmic reticulum (Terasaki et al., 1991), and once more in a modified PKME containing NaCl in place of KCl. The dishes were examined microscopically to ensure the complete removal of all eggs and egg fragments.

CVs were removed from isolated planar cortices by squirting a forceful stream of IM at the cortex using a syringe and a 25-gauge needle, followed by careful washing with additional IM buffer and then with PKME and modified PKME (NaCl buffer). Completeness of CV removal was assessed using phase contrast microscopy. In addition, quantitative western blots for two CV cargo proteins (K.T. and J.Z., unpublished) and for calreticulin (Affinity BioReagents, antibody #PA3-900) were used to determine the extent of removal of CVs and adherent endoplasmic reticulum, respectively (see Results).

For electrophoresis, IPC and vesicle-denuded IPC were dissolved, after aspiration of buffer, by the addition of 1% SDS sample buffer containing 6% sucrose, 0.05 M $NaPO_4$ at pH 7.5, 0.10 M dithiothreitol, 3 mM EDTA and 0.5 mM AEBSF (Pefabloc SC; Boehringer Mannheim; added just prior to use).

Isolation and fusion activity of cortical vesicles

CVs were prepared by a variation of the method of Crabb and Jackson (Crabb and Jackson, 1985), as previously described (Coorssen et al., 1998; Tahara et al., 1998). CSCs were prepared by homogenization of eggs in IM and, after washing in the same buffer, the CSCs were transferred on ice to a buffer consisting of 450 mM KCl, 10 mM EGTA, 10 mM BAPTA, 50 mM NH_4Cl, 1 mM benzamidine HCl, 5 mM DTT, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 20 μg/ml leupeptin, pH 9.1. After 1 hour many vesicles had detached from the plasma membrane. CVs were purified from the plasma membrane sheets by centrifugation at 700 ***g*** for 5 minutes at 4°C. The supernatant containing the CVs was retained and this centrifugation was repeated. Finally, the CVs were collected by centrifugation at 2000 ***g*** for 10 minutes at 4°C, resuspended in a small volume of IM buffer on ice and retained at –80°C for western blotting. The concentration of CV in this suspension was determined by counting in a hemocytometer under phase contrast illumination. The fusion competence of preparations used in this study was determined by producing Ca^{2+} activity curves as previously described (Coorssen et al., 1998).

Preparation of egg cytosol

Eggs were dejellied and washed as above and washed twice more with ice cold IM. The eggs were resuspended in an equal volume of IM and were gently homogenized by three strokes of a loose-fitting Teflon pestle in a Potter homogenizer. The homogenate was centrifuged for 1 hour at 4°C and 100,000 ***g***. Cytosol was stored at –80°C until use.

Immunoabsorption of NSF from cytosol

Cytosol was incubated with polyclonal sea urchin NSF antibody (50 μg/ml) for 1 hour at room temperature with gentle agitation. Following this, the cytosol was incubated for 30 minutes with Protein G Sepharose (50 μl packed beads/ml) with gentle agitation. The mixture was centrifuged at 10,000 ***g*** for 2 minutes to pellet the beads, and the cytosol supernatant was removed. The NSF content of cytosol and immuno-absorbed cytosol was determined by electrophoresis and western blotting.

Binding of NSF to isolated CVs

CVs were prepared as above and were incubated with cytosol at a concentration of 1 mg/ml for 1 hour at room temperature with gentle mixing. Where appropriate, the cytosol was supplemented with 2 mM ATP or 200 μM ATP-γ-S. After incubation, the CVs were washed by centrifugation with excess IM buffer containing the appropriate nucleotide (if any). Washing was repeated three times, after which CVs were dissolved in sample buffer prior to electrophoresis and western blotting.

Electrophoresis and western blotting

Following SDS-PAGE, proteins were transferred overnight to 0.45 μm PVDF membranes, and blots were blocked for 30 minutes at room temperature in a solution consisting of 140 mM NaCl, 10 mM $NaPO_4$, 0.05% Tween 20, 50 mg/ml bovine serum albumin, pH 7.4. Blots were incubated overnight at 4°C with anti-NSF antibodies diluted in blocking solution containing 20 mg/ml bovine serum albumin. Proteins were visualized using alkaline phosphatase-linked second antibody, enhanced chemifluorescence (Amersham International, Amersham, UK), and Storm System fluorescence imaging and quantitation (Molecular Dynamics, Sunnyvale, CA). Pre-stained molecular mass markers were from Novex (San Diego, CA).

Isolation of sea urchin NSF clones

Two clones carrying incomplete sea urchin NSF sequences were isolated by expression screening of a *S. purpuratus* ovary cDNA library in λZAP (Stratagene, LaJolla, CA) using monoclonal antibody 6E6, horseradish peroxidase-linked second antibody and enhanced chemiluminescence. The library was kindly supplied by Dr G. Wessel (Laidlaw and Wessel, 1994). The positive clones were plaque-purified by iterative screening and the insert was excised in vivo according to the supplier's instructions (Stratagene). The sequences of the two clones were identical in the region of overlap (~80% of their length). The sequence data were used to design primers for amplification of the 5′ end of NSF (and later, of full-length NSF) from a *S. purpuratus* two-hybrid library (Clontech) using Advantage PCR kits and T-A cloning kits (Clontech). Full-length PCR product was subcloned into pBAD-TOPO (InVitroGen) for expression.

Purification and characterization of contents proteins

We identified several soluble proteins that were highly enriched in CVs, were not present in cytosol and were readily released from CVs by freeze-thawing followed by high-speed centrifugation to remove membranes. After partial purification, the proteins were subjected to Edman microsequencing. Parts of the resulting N-terminal peptide sequences (ERTIAFTVERTADMSDSTTSR and SEFLNDFGLSIL-DKALT) were used to generate peptides that were linked to keyhole limpet hemacyanin. Injection into rabbits resulted in production of polyclonal antibodies that were affinity purified and that detected

single bands in western blots of CV proteins (K.T. and J.Z., unpublished). The cDNAs for the two proteins were cloned and the accession numbers are AF172720 and AF172721.

Recombinant NSF protein standards

Recombinant Chinese hamster ovary NSF-myc-his_6 (epitope-tagged NSF) was purified from a lysate of *E. coli* using a Ni^{2+} affinity column (HiTrap Chelating) and was 97% pure as determined by densitometric scanning of a Coomassie Blue-stained SDS polyacrylamide gel. Approximately 4 mg of epitope-tagged Chinese hamster NSF was dialyzed against dilute NH_4HCO_3 buffer and lyophilized. The dry protein was weighed out (2.00±0.05 mg) and dissolved in an SDS-containing buffer. Recombinant sea urchin NSF-his_6 was similarly purified from *E. coli* to 80% purity. Its concentration was measured by densitometric scanning of the NSF band in a Coomassie Blue-stained SDS gel in comparison with a known amount (300 ng) of Chinese hamster NSF run in the same gel.

Results

CV exocytosis is unaffected by non-hydrolyzable ATP analogs

It has previously been demonstrated that CV exocytosis does not require ATP hydrolysis (Moy et al., 1983; Whalley and Whitaker, 1988) and is not inhibited by non-hydrolyzable ATP analogues in vitro (Whalley et al., 1991), at least in the short term. However, it has been suggested that ATP might play a modulatory role in maintaining Ca^{2+} sensitivity (Baker and Whitaker, 1978; Sasaki and Epel, 1983). We have assessed the effects of long incubations with non-hydrolyzable ATP analogues on CV exocytosis in vitro. CSCs were prepared in IM and were incubated with the specified nucleotides for 90 minutes. Ca^{2+}-dependent exocytosis was then measured (Fig. 1). No effect could be detected on the Ca^{2+} sensitivity or the extent of CV exocytosis by either ATP, the poorly hydrolyzable analog ATP-γ-S or the non-hydrolyzable analog AppNHp.

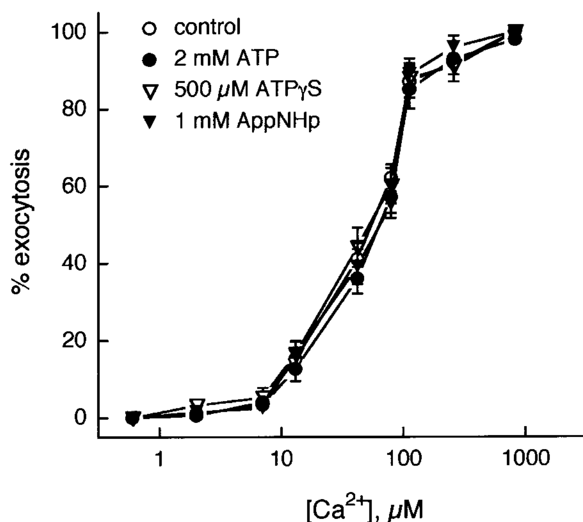

Fig. 1. Non-hydrolyzable analogues of ATP have no effect on CV exocytosis in vitro. CSCs were prepared in IM and were incubated in the absence of nucleotides (open circles) or with either ATP (closed circles), the poorly hydrolyzable analogue ATP-γ-S (open triangles) or the non-hydrolyzable analogue AppNHp (closed triangles) for 90 minutes at room temperature (20°C) with gentle mixing. Ca^{2+}-dependent exocytosis was then measured in the continued presence of the added nucleotides. Mean±s.e.m. are shown, n=3.

Inhibition of CV exocytosis by thiol reagents is not reversed by soluble proteins

CV exocytosis is inhibited by compounds such as NEM that modify cysteine residues (Haggerty and Jackson, 1983; Vogel et al., 1992; Whalley and Sokoloff, 1994) and in this respect is similar to many other intracellular membrane fusion processes (Rothman, 1994). The sites that are modified by thiol-reactive agents are likely to be in an exposed environment; in the species *Lytechinus pictus*, CV exocytosis can be inhibited by large and/or hydrophilic molecules such as thiol-reactive dextrans (Whalley and Sokoloff, 1994) and AMSDS. In the present study, we have confirmed and extended this finding using CSCs from *S. purpuratus* eggs, using conditions in which exocytotic fusion was only partially inhibited. When CSCs were treated with 200 μM D10PDP for 1 hour prior to triggering exocytosis, some exocytosis still persisted with the highest concentrations of Ca^{2+} (Fig. 2A). When CSCs were treated with 1 mM NEM, the Ca^{2+} required for exocytosis was increased but the extent of exocytosis was only partially reduced. Higher concentrations of both D10PDP and NEM gave complete inhibition of fusion (data not shown). In contrast, after incubation with 50 μM AMSDS, CV exocytosis was completely abolished even at the highest Ca^{2+} concentration tested (Fig. 2A).

Next, we assessed the ability of egg cytosol to reverse the inhibition of fusion by these treatments. CSCs were suspended in DTT-free IM and were treated with no addition, 1 mM NEM, 200 μM D10PDP or 50 μM AMSDS for 1 hour with gentle agitation. Following these treatments, CSCs were washed extensively by centrifugation and resuspension in IM containing DTT, to remove all traces of the thiol-reactive compounds, and then were incubated with cytosol at a protein concentration of 10 mg/ml for 30 minutes. The NSF concentration in this cytosol was determined to be ~4.4 μg/ml. Exocytosis was measured in response to Ca^{2+}. As can be seen in Fig. 2B (compared with Fig. 2A), the addition of cytosol had no restorative effect on fusion inhibited by any of these thiol-specific reagents, whether inhibition was partial or complete.

Cloning of sea urchin NSF and production of a functionally inhibitory antibody

In order to determine any physiological role for NSF in CV fusion it was necessary to develop a species-specific, functionally inhibitory antibody. It was also necessary to produce such an antibody to determine quantitatively, and with maximum sensitivity, whether either the isolated egg cortex or CV membranes contain any NSF protein. This necessitated cloning the protein in order to determine a suitable epitope. Because certain monoclonal antibodies to Chinese hamster NSF are able to recognize cytosolic sea urchin NSF (data not shown), these antibodies were used to screen an expression library in λZAP. Those studies produced two incomplete, overlapping clones that spanned both ATP-binding domains (537 encoded amino acids). Subsequent studies using a

different cDNA library and rapid amplification of cDNA ends (RACE) led to the complete sequence. The encoded amino acid sequence (Fig. 3) contains 744 residues, constituting a polypeptide with a M_r of 82,402. Homology between the sea urchin and Chinese hamster proteins is high, with 65% identical and 73% similar amino acid residues. A 22-residue peptide corresponding to the D1-D2 border (underlined in Fig. 3) was chosen as the basis for a peptide-directed antibody, which was affinity purified before use. We tested the specificity of the antibody using Western blotting of purified sea urchin egg cytosol. This showed that there were two major bands that reacted with the antibody (Fig. 4A). The first had an apparent molecular mass of ~78 kDa and the second had an apparent molecular mass of ~58 kDa. If the antibody was pre-incubated with a three-fold molar excess of the immunizing peptide, it failed to recognize the upper band although could still recognize the lower band (Fig. 4B). The mobility of the upper band in cytosol was identical to that of a sample of partially purified sea urchin NSF and the antibody also could recognize recombinant Chinese hamster NSF (not shown). The use of this antibody in western blots resulted in an 8- to 10-fold increase in sensitivity for the detection of sea urchin NSF as compared to monoclonal antibody 6E6 (data not shown). Thus this antibody is useful in recognizing NSF in sea urchin egg samples.

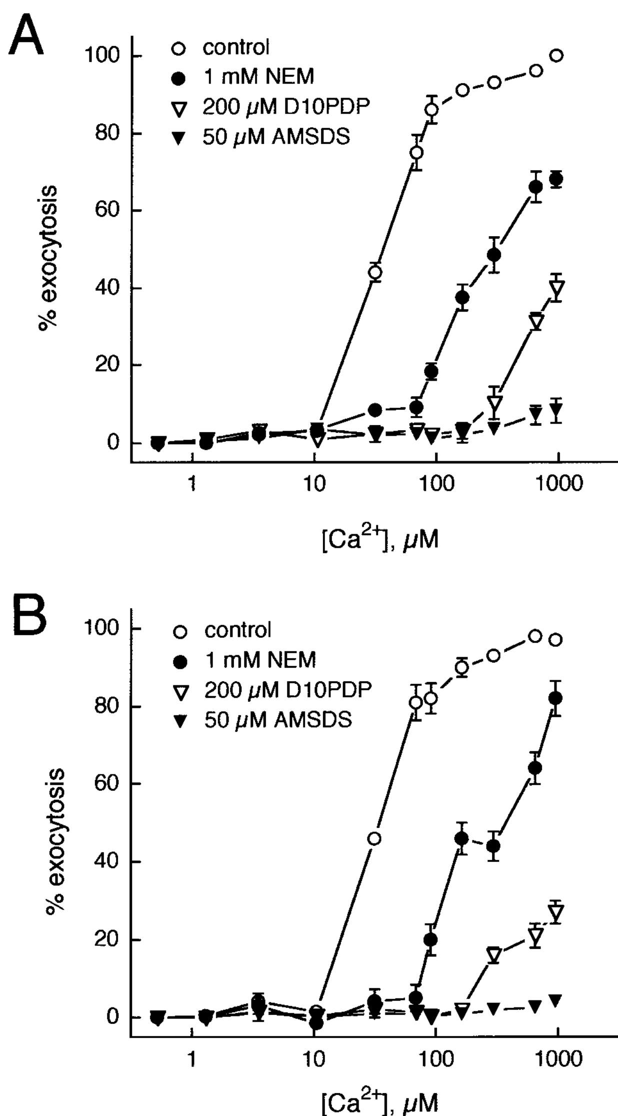

Fig. 2. Soluble proteins do not reverse inhibition of CV exocytosis by thiol reagents. (A) CSCs were prepared in DTT-free IM and were treated for 1 hour at room temperature (20°C) with gentle mixing with the following. No thiol reagent (open circles), 1 mM NEM (closed circles), 200 μM D10PDP (open triangles) or 50 μM AMSDS (closed triangles). The CSC were centrifuged at 700 ***g*** for 1 minute and suspended in fresh IM before the addition of Ca^{2+} and measurement of exocytosis. Mean±standard error of the mean are shown, *n*=3 except for AMSDS where mean±standard deviation are shown, *n*=2. (B) After the treatment described in A, the CSC were washed by a further centrifugation step and were incubated with cytosol at a protein concentration of 10 mg/ml (~0.1 μg NSF/mg protein) and 5 mM ATP for 30 minutes. Ca^{2+} was added and exocytosis measured. Mean±s.e.m. are shown, *n*=3 except for AMSDS where mean±s.d. are shown, *n*=2.

The antibody was tested for its ability to react with native sea urchin NSF. A fraction of cytosol was incubated with the antibody at a final concentration of 50 μg/ml. After 1 hour at room temperature, the mixture was incubated with Protein A-Sepharose. Following centrifugation, the supernatant was subjected to SDS-PAGE and western blotting using the species-specific anti sea urchin NSF antibody. The western blot analysis indicated that cytosol incubated with anti-NSF antibody was depleted of NSF showing that the antibody was capable of binding to the native, folded and functional protein (data not shown).

NSF binding sites are present in the exocytotic apparatus

We wanted to determine whether NSF could interact with the sea urchin egg exocytotic apparatus as this might indicate a role for NSF in CV priming, CV fusion or in membrane recycling following exocytosis (Morgan and Burgoyne, 1995; Banerjee et al., 1996). We tested the ability of NSF to bind to CV and PM by developing an NSF-binding assay, taking advantage of the stabilization of NSF hexamers in the presence of non-hydrolyzable analogs of ATP (Wilson et al., 1992). The number of binding sites was measured by incubating CVs with NSF-containing egg cytosol in the presence of 200 μM ATP-γ-S, followed by a single wash in IM buffer containing the same nucleotide. Under these conditions, egg NSF bound to CV membranes (Fig. 5A, lanes 5,6), resulting in 11.1±1.2 copies of NSF hexamer bound per CV (mean±s.e.m. for two experiments in duplicate). This binding of NSF did not prevent CV fusion with identical Ca^{2+} sensitivity as untreated CVs (data not shown). Less binding was evident in the absence of added nucleotide (lanes 3 and 4; 6.8±1.6 copies per CV) or with cytosol supplemented with ATP (Fig. 5A, lanes 7 and 8, 1.2±0.2 copy per CV), probably because most of the NSF that may have been bound initially was removed during the wash with protein-free, Mg^{2+}-containing buffer. This behavior is consistent with retention of activity of egg cytosolic NSF, since replacement of ATP-γ-S by ATP in the wash buffer would allow exchange and hydrolysis of ATP, leading to dissociation from SNARE binding sites (Söllner et al., 1993). In addition, partial purification of egg cytosol NSF according to the procedure of Block et al. (Block et al., 1988) resulted

in NSF sedimenting in the ultracentrifuge as a hexamer in the presence of ATP (data not shown), indicating retention of its native configuration. Epitope-tagged Chinese hamster NSF also binds to IPC in the presence of α-SNAP and ATP-γ-S (not shown).

NSF antibody prevents NSF binding to the exocytotic apparatus

We used the NSF binding assay described above to determine whether the NSF antibody was functionally inhibitory. IPCs were prepared on polylysine-coated Petri dishes and were incubated at room temperature with cytosol at a concentration of 2 mg/ml supplemented with 200 μM ATP-γ-S. In addition, each preparation contained the NSF antibody at concentrations from 0 to 40 μg/ml. After 1 hour, dishes were extensively rinsed with IM containing 200 μM ATP-γ-S and IPCs dissolved in sample buffer. Western blotting was used to assess NSF binding. As can be seen (Fig. 5B) there was little effect of the antibody on NSF binding at concentrations of 0.5 and 2 μg/ml. However, incubation of cytosol with 10 μg/ml antibody significantly reduced NSF binding and NSF binding was abolished when cytosol was incubated with the antibody at a concentration of 40 μg/ml. These data clearly indicate that the antibody is capable of binding to the native protein, and that it is functionally inhibitory.

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Sea Urchin  1     MSHTREMKAVRCPTDQLSLTNRAVVSDKDAFARIDYVQVQAIPGPSYVFA    50
Hamster     1     MAG-RSMQAARCPTDELSLSNCAVVSEKD-YQSGQHVIVRTSPNHKYIFT    48
                  *:  *.*:*.*****:***:* ****:** :    ::* *:: *. .*:*:

Sea Urchin  51    TVAHPDLKSGEMGFSLPQRKWARISIDQPIQAAPYRFDPSCQYLSSITVE    100
Hamster     49    LRTHPSVVPGSVAFSLPQRKWAGLSIGQEIEVALYSFDKAKQCIGTMTIE    98
                  :**.: .*.:.********* :**.* *:.* * ** : * :.::*:*

Sea Urchin  101   VDFLQKKNTTNDAFDSDKMAKDFLESFDRVAFSVEQQLVCSFYNKKLMGL    150
Hamster     99    IDFLQKKNIDSNPYDTDKMAAEFIQQFNNQAFSVGQQLVFSFN-DKLFGL    147
                  :*******  .:.:*:**** :*::.*:. **** **** **  .**:**

Sea Urchin  151   VVKSVEILDPAILKGGKPSAKRNRDVRIGVLTQNTQVTFEKAEGSALNLT    200
Hamster     148   LVKDIEAMDPSILKGEPASGKRQK-IEVGLVVGNSQVAFEKAENSSLNLI    196
                  :**.:* :**:****  .*.**:: :.:*::. *:**:*****.*:***

Sea Urchin  201   GRSKGQSGNQSIINPDWDFKKMGIGGLDKEFSDIFRRAFASRVFPQEFVE    250
Hamster     197   GKAKTKENRQSIINPDWNFEKMGIGGLDKEFSDIFRRAFASRVFPPEIVE    246
                  *::* :...*******:*:************************* *:**

Sea Urchin  251   QIGAQHVKGILLFGPPGTGKTLMARTIGKMLNSREPQIINGPEILNKFVG    300
Hamster     247   QMGCKHVKGILLYGPPGCGKTLLARQIGKMLNAREPKVVNGPEILNKYVG    296
                  *:*.:*******:**** ****:** ******:***::*********:**

Sea Urchin  301   ESEANIRKLFAAAEDEQKKMGNNSGLHIIIFDEIDAICKQRGSMSGSTGV    350
Hamster     297   ESEANIRKLFADAEEEQRRLGANSGLHIIIFDEIDAICKQRGSMAGSTGV    346
                  *********** **:**::* ***********************:*****

Sea Urchin  351   HDTVVNQLLSKIDGVEQLNNVLLIGMTNRKDLIDDALLRPGRLEVQMEIG    400
Hamster     347   HDTVVNQLLSKIDGVEQLNNILVIGMTNRPDLIDEALLRPGRLEVKMEIG    396
                  ********************:*:****** ****:**********:****

Sea Urchin  401   LPDEAGRLQIIEIYLAKMKENGKLSKDVDPMELSTLTKNYSGAEIAGLVR    450
Hamster     397   LPDEKGRLQILHIHTARMRGHQLLSADVDIKELAVETKNFSGAELEGLVR    446
                  **** *****:.*: *:*: :  ** ***  **:. ***:****: ****

Sea Urchin  451   AAQSMAMNSLITASSKVEVDYEKVEKMQVSREHFMHAMDTDIKPAFGVSS    500
Hamster     447   AAQSTAMNRHIKASTKVEVDMEKAESLQVTRGDFLASLENDIKPAFGTNQ    496
                  **** ***  *.**:***** **.*.:**:* .*: :::.*******...

Sea Urchin  501   ENFDKFILNGIIDWGEPVQRVLADGELVVKQTKASDRVPLASMLMGGRSG    550
Hamster     497   EDYASYIMNGIIKWGDPVTRVLDDGELLVQQTKNSDRTPLVSVLLEGPPH    546
                  *:: .:*:****.**:** *** ****:*:*** ***.**.*:*: * .

Sea Urchin  551   SGKTALAAKLALESGFPFIKICSAEDMVGFSESAKCQAIKKIFEDAYKSQ    600
Hamster     547   SGKTALAAKIAEESNFPFIKICSPDKMIGFSETAKCQAMKKIFDDAYKSQ    596
                  *********:* **.********.:.*:*****:*****:****:******

Sea Urchin  601   VSCVVVDDIERLLDYVPIGPRFSNLVLQALLVLLKKAPPKGRKLLIIGTT    650
Hamster     597   LSCVVVDDIERLLDYVPIGPRFSNLVLQALLVLLKKAPPQGRKLLIIGTT    646
                  :**************************************:**********

Sea Urchin  651   SAHDVLEEMKMTDIFNTYVRVNSLSTSEHIVKVLEELKIFTPEERERIRT    700
Hamster     597   SRKDVLQEMEMLNAFSTTIHVPNIATGEQLLEALELLGNFKDKERTTIAQ    696
                  * :***:**:* : *.* ::* .::*.*::::.** *  *. :**  *

Sea Urchin  701   KTQGKSLLIGIKKLLVLLEMVRQTEPSYRVPKFLSLMEEEGGLELS--      746
Hamster     697   QVKGKKVWIGIKKLLMLIEMSLQMDPEYRVRKFLALLREEGASPLDFD      744
                  :.:**.: *******:*:**  * :*.*** ***:*:.***.  *.
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Fig. 3. Alignment of amino acid sequences of sea urchin NSF and Chinese hamster NSF. GenBank accession numbers: sea urchin NSF AF171096; Chinese hamster, X15652. An asterisk (*) underneath the sequences indicates amino acid identity, two dots (:) a conservative substitution and a single dot (.) a semi-conservative substitution. Residues in bold are putative nucleotide-binding motifs within each domain (Walker et al., 1982) at residues 264-271 and 547-554 of the sea urchin sequence. The underlined peptide sequence, which spans the D1-D2 domain boundary at residue 490 (Whiteheart et al., 1994), was used to generate polyclonal antibodies.

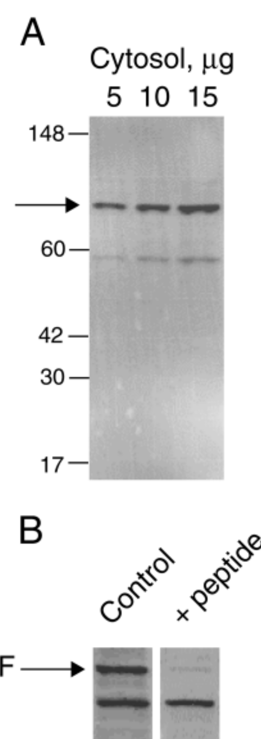


Fig. 4. Antibody recognizes sea urchin egg cytosol NSF and is inhibited by the immunizing peptide. (A) Affinity-purified anti-NSF antibody was tested against sea urchin egg cytosol by western blotting. 5, 10 or 15 μg of cytosol was subjected to electrophoresis as indicated. Following transfer onto PVDF, the blot was incubated with anti-NSF at a concentration of 1:2000 (~0.5 μg/ml) overnight at 4°C. Bound antibody was visualized using alkaline phosphatase-linked second antibody, enhanced chemifluorescence and Storm system fluorescence imaging. The position of putative NSF is indicated with the arrow. (B) 10 μg of cytosol was subjected to electrophoresis and western blot analysis as above but in the presence and absence of the immunizing peptide at a ratio of 3:1 (peptide:IgG). The NSF antibody was incubated on ice for 1 hour with the immunizing peptide prior to incubation with the blot. The labeled arrow indicates the position of the NSF band.

Anti-NSF antibodies do not inhibit CV exocytosis or homotypic fusion

The ability of the antibody to bind to and inactivate native NSF was used to probe the physiological involvement of NSF in CV exocytosis and fusion. CSCs were prepared and incubated with the antibody or with a control antibody directed against hyalin, one of the content proteins of the CVs. Incubation proceeded for 1 hour at room temperature with gentle mixing after which time the Ca^{2+} sensitivity of exocytosis was determined (Fig. 6A). As can be seen, the presence of anti-NSF had no effect on Ca^{2+}-stimulated exocytosis. The failure of the antibody to affect the Ca^{2+} sensitivity of exocytosis suggests that NSF is not involved in this process. However, electron microscopic analysis of CV/PM contact areas (Chandler and Heuser, 1979) and the stable nature of in vitro PM/CV association (Vacquier, 1975), suggests a tight juxtaposition of the two membranes making it possible that antibodies would not have access to the fusion site. To overcome the possible problem of antibody accessibility, we tested NSF antibodies in homotypic CV fusion assays where accessibility is not limited by pre-existing membrane contacts. As can be seen in Fig. 6B, NSF antibodies failed to have any inhibitory effect on CV-CV fusion suggesting that NSF does not have a role in this Ca^{2+}-triggered membrane fusion.

Membrane fusion and NSF are differentially sensitive to NEM

Previous studies of CV exocytosis and homotypic fusion (Haggerty and Jackson, 1983; Vogel et al., 1992; Whalley and Sokoloff, 1994) have shown that the sensitivity of sea urchin egg membrane fusion to NEM is less than that of mammalian in vitro fusion assays in which NSF plays a clear role (Glick and Rothman, 1987). In yeast too, the sensitivity of Sec18 to NEM is much reduced compared to that of mammalian NSF (Wilson et al., 1989; Haas and Wickner, 1996). This is thought to be due to the substitution of a threonine (Thr285) for the primary NEM target in mammalian NSF, Cys264, that is present in the ATP-binding pocket of the D1 loop (Tagaya et al., 1993). An analysis of the sequence of sea urchin NSF (Fig. 3) shows that here too there is a substitution in the D1 loop of a cysteine residue with a threonine (Thr266). This observation suggests that sea urchin NSF is unlikely to be sensitive to NEM or other thiol-reactive agents, or at least that sensitivity should be much reduced compared to mammalian NSF. Is it possible that the reduced sensitivity of CV exocytosis towards NEM reflects that the target for NEM in NSF is other than a cysteine

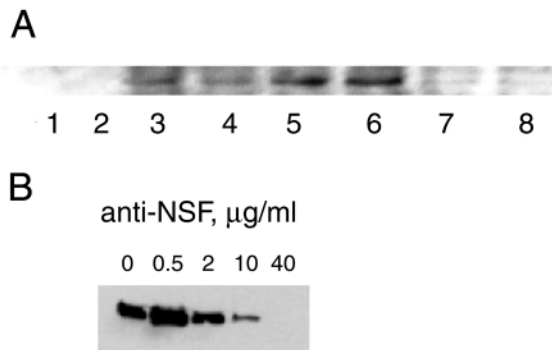
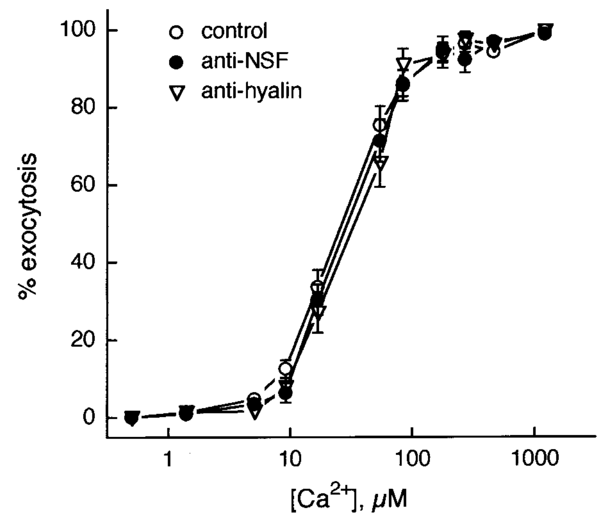


Fig. 5. Cytosolic NSF binds to egg membranes and is inhibited by NSF antibodies. (A) CVs were incubated with cytosol, and with and without (±) nucleotides for 1 hour at room temperature followed by washing in IM ± appropriate nucleotides. Lanes 1, 2, CVs alone; lanes 3, 4, CVs incubated with cytosol without added nucleotide; lanes 5, 6, CVs incubated with cytosol supplemented with 200 µM ATP-γ-S; lanes 7, 8, CVs incubated with cytosol supplemented with 2 mM ATP. (B) IPCs were incubated at room temperature with cytosol at a concentration of 2 mg/ml supplemented with 200 µM ATP-γ-S. NSF antibody was included at concentrations from 0 to 40 µg/ml as indicated. After 1 hour, dishes were extensively rinsed with IM plus 200 µM ATP-γ-S and IPCs dissolved in sample buffer. NSF binding was visualized by western blotting.



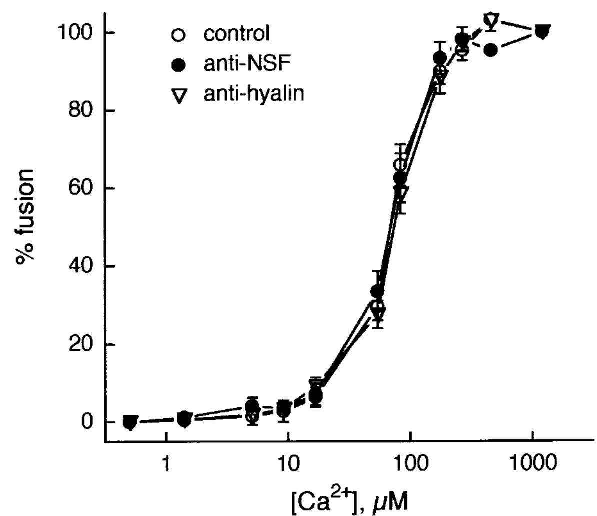


Fig. 6. Anti-NSF antibodies do not affect membrane fusion. (A) CSCs were incubated with no antibody (open circles), 50 µg/ml anti-NSF (closed circles) or 50 µg/ml anti-hyalin (open triangles). After 1 hour incubation at room temperature, Ca^{2+} sensitivity of exocytosis was determined. Mean±standard error of the mean are shown, n=3. (B) CVs were incubated with no antibody (open circles), 50 µg/ml anti-NSF (closed circles) or 50 µg/ml anti-hyalin (open triangles). After 1 hour incubation at room temperature Ca^{2+} sensitivity of CV/CV fusion was determined. Mean±s.e.m. are shown, n=3.

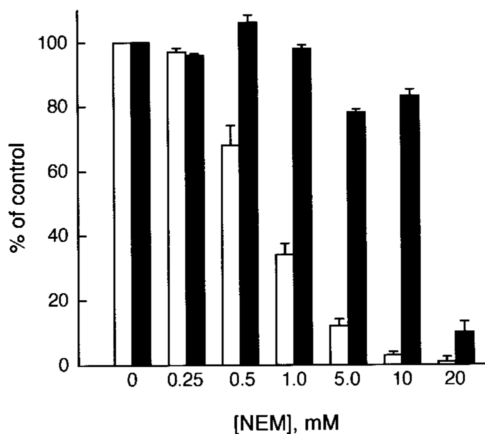


Fig. 7. CV fusion and NSF binding are differentially sensitive to NEM. Exocytosis in isolated cortices was determined following incubation with various concentrations of NEM for 1 hour at room temperature. Sensitivity of exocytosis to 100 μM Ca^{2+} was then assessed (open bars). Mean±s.e.m. are shown, *n*=3. The binding of NSF was determined by incubating cortices with cytosol and 100 μM ATP-γ-S. Prior to the binding assay, cytosol was treated for 1 hour at room temperature with the same concentrations of NEM used to inhibit CV exocytosis. NSF binding was determined by western blotting with anti-NSF antibody followed by densitometric scanning (filled bars).

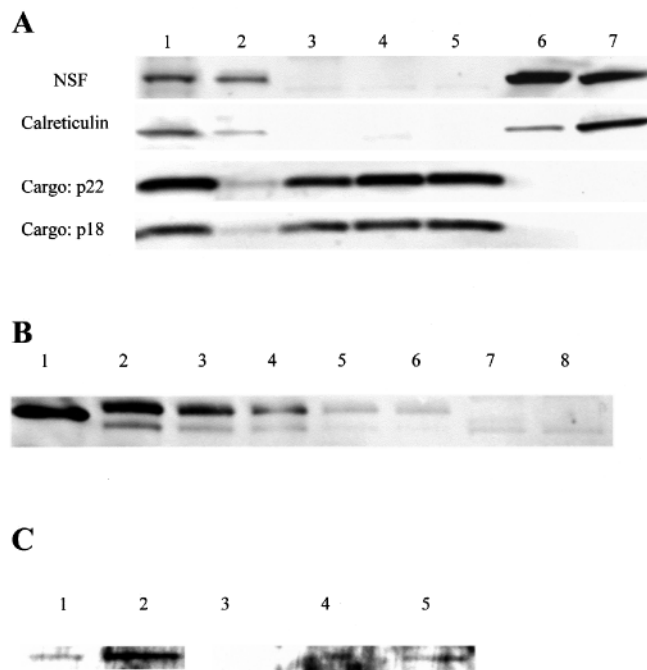


Fig. 8. NSF is present in isolated planar cortex but is not associated with CVs. (A) Replicate western blots probed for sea urchin NSF, calreticulin and two CV cargo proteins. Lane 1, IPC containing 1.5×10^7 CVs; lane 2, an equivalent amount of cortex denuded of CVs; lanes 3-5, 1.5×10^7 isolated CVs (three different preparations); lane 6, egg cytosol (5 μg total protein); lane 7, egg microsomes (10 μg total protein). (B) NSF western blot. Lane 1, egg cytosol (5 μg total protein); lanes 2-6, recombinant NSF-His_6 standard curve (0.48, 0.24, 0.12, 0.048, and 0.024 fmol as hexamer); lanes 7, 8, two preparations of 1.5×10^7 isolated CVs. (C) Recovery in western blots of recombinant sea urchin NSF added to cortical vesicle material. Lane 1, 0.024 fmol NSF alone; lane 2, 0.048 fmol NSF; lane 3, CV proteins; lane 4, CV proteins plus 0.024 fmol NSF; lane 5, CV proteins plus 0.048 fmol NSF. Representative of two experiments.

residue in the D1 loop, or does it indicate that the target is a completely different protein? Because we have a functional assay for NSF in its ATP-dependent binding to exocytotic membranes, we were able to test this finding directly. We assayed the sensitivity of CV exocytosis and NSF binding to inhibition by NEM. Exocytosis in isolated cortices was determined following incubation with various concentrations of NEM for 1 hour at room temperature. At the same time, the binding of NSF to cortices was determined by incubating cortices with cytosol and 100 μM ATP-γ-S. Prior to the binding assay, cytosol was treated for 1 hour at room temperature with the same concentrations of NEM used to inhibit CV exocytosis. The results of these experiments are shown in Fig. 7. As can be seen, there is a clear difference between the sensitivity of CV exocytosis and that of NSF binding towards NEM. There is no effect on the binding of NSF to cortical membranes except at 20 mM NEM, the highest concentration tested. In contrast to this, CV exocytosis starts to be inhibited by incubation with 0.5 mM NEM, and is 90% inhibited by 5 mM NEM. These data suggest that the protein target of NEM that inhibits CV exocytosis is distinct from NSF.

NSF is associated with the PM but not with CV

All of the data presented so far are consistent with the hypothesis that fusion of CVs proceeds in the absence of NSF. However, it is still possible that NSF might participate in CV fusion in a manner that is independent of its ATPase activity and is insensitive to inhibition by thiol-reactive agents. In order to rule out this possibility we performed a quantitative analysis to determine whether or not stoichiometric quantities of NSF are present on CVs. We analyzed samples of isolated planar cortex, isolated PM and purified CVs for the presence of NSF using western blotting. Samples of isolated planar cortex (IPC) prepared for use in immunoblot analysis were free of cytosol and whole eggs, as described in the Materials and Methods. The number of CVs was estimated based on the area of the cortex, allowing 1 μm^2 per CV. Additional samples consisted of IPC from which ~90% of the CVs had been removed by mechanical shear force (with a stream of IM), and CVs isolated by high pH treatment from cell surface complexes and counted accurately in a hemocytometer. Apparent interference in NSF western blots by other, co-migrating CV proteins was observed in preliminary experiments and was minimized by limiting the total protein loaded per lane to 15 μg or ~1.5×10^7 isolated CVs. Fig. 8A shows that, using the species-specific antibody, NSF was clearly detected in IPC. We estimated the number of copies of hexameric NSF at 8.4±2.3 per square μm, corresponding to ~8/CV. However, removing CVs and ER from the cortices did not remove much NSF (mean loss of 26%±2.1%), despite a marked reduction of both the ER marker calreticulin (loss of 80±7%) and CV cargo proteins (loss of 79±4%), in three experiments. Because of the removal of CV proteins, the content of NSF per mg protein was 1.5- to 3.1-fold higher in vesicle-denuded membranes (40±25 fmol/mg protein) than in intact cortices (14.3±4.0 fmol/mg protein, *n*=3 paired, independent experiments).

In contrast to samples containing PM, NSF content in samples of isolated CVs was very low and often undetectable by western blotting (Fig. 8A,B), despite documented fusion competence with normal Ca^{2+} dependence in the same vesicle preparations (data not shown). Fig. 8B shows one experiment in which 0.024 fmol (calculated as hexamer) of recombinant sea urchin NSF-His_6 was easily detectable, while in the same immunoblot NSF could not be detected in samples of CV proteins. These data are representative of four experiments using a total of five different preparations of CVs. Fig. 8C shows experiments in which 0.024 and 0.048 fmol of added NSF (as internal standards) were each still detectable in the presence of CV proteins, with an estimated recovery of 45±9%.

In these experiments, 0.024 fmol of NSF hexamer corresponds to 1 copy per CV. The signal in CV samples (when present) was always smaller than this. Nevertheless, analysis with ImageQuant software showed that a very faint band at the position of authentic, cytosolic NSF was sometimes present, equivalent to 0.168±0.061 (mean±s.e.m., n=10) copies per CV (range, 0.0-0.50), or 2% as much as in IPC per milligram protein. In addition, there were other, also very weak, cross-reacting bands of both higher and lower electrophoretic mobilities. The reactive bands with higher mobilities are unlikely to be proteolytic products of NSF and the addition of exogenous NSF to CV samples prior to sample preparation and electrophoresis did not increase the levels of these minor bands (data not shown). The slight degree of cross reaction with other CV proteins was probably due to the use of the peptide-directed antibody in highly concentrated form, which was necessary to ensure detection of 0.024 fmol (12.1 pg) per lane. Thus, the presence of very weakly reactive bands in the vicinity of that corresponding to cytosolic NSF may well have been due to minor cross reaction of the antibody with a different, co-migrating protein. Notwithstanding this, there was no evidence, in any gel, for one full copy of NSF hexamer per CV, arguing against a correlation between NSF density and the ability of CV to engage in Ca^{2+}-triggered fusion. Similar experiments using monoclonal antibody 6E6 also failed to detect any NSF protein in isolated CV, though with a 10-fold higher detection limit (data not shown).

Discussion

At fertilization in sea urchin eggs, CV exocytosis is rapidly triggered (Chandler, 1991; Swann and Whitaker, 1986) and is followed by membrane retrieval (Whalley et al., 1995). That the CVs are docked to the PM in the unfertilized egg can be demonstrated by the ability to purify an intact exocytotic machine (either CSC or IPC) in which the PM and CVs remain tightly associated (Vacquier, 1975). These CVs are resistant to shear stress (Vacquier, 1975; Whalley et al., 1991; Coorssen et al., 1998) and are docked by ultrastructural criteria (Chandler, 1991; Chandler, 1984; Sardet, 1984). In this work we have used a combination of physiological and biochemical techniques to show that one protein, NSF, does not play a role in either CV exocytosis or in CV-CV fusion.

CV exocytosis is susceptible to inhibition by NEM (Haggerty and Jackson, 1983; Vogel et al., 1992; Jackson and Modern, 1990) and other thiol-specific reagents (Whalley and Sokoloff, 1994), which indicates the association of thiol-containing proteins with the secretory process. However, for a number of reasons, the target protein for these inhibitors is unlikely to be NSF, a cytosolic ATPase thought to be important in many intracellular membrane fusion events (Rothman, 1994). Firstly, soluble proteins are not required for CV exocytosis (Whalley et al., 1991; Vogel et al., 1992) and do not stimulate or alter the Ca^{2+}-dependence of exocytotic fusion. This shows that the fusing CV and PM contain all of the necessary protein machinery for Ca^{2+} sensing and fusion in tightly bound form, unlike Golgi and other in vitro fusion systems that require the addition of cytosolic elements (Block et al., 1988). However, in each of these cytosol-dependent fusion systems, membrane fusion is the last event of a multi-step process that may include vesicle formation, vesicle translocation and vesicle-target membrane docking. Any or all of these other events might require the chaperone-like activity of NSF (Morgan and Burgoyne, 1995).

Secondly, CV exocytosis, like other forms of triggered exocytosis (Howell et al., 1987; Holz et al., 1989; Heidelberger, 1998), does not require ATP in order to proceed through triggering and fusion (Moy et al., 1983; Whalley and Whitaker, 1988). This makes the involvement of an ATPase in this reaction unlikely. While the rate of hydrolysis of ATP by NSF suggests that any pre-bound ATP should be hydrolyzed within a few minutes of incubation in vitro (Tagaya et al., 1993), it is possible that tightly bound, protected ATP might be made available to an ATPase upon triggering fusion. However, we have shown here that lengthy (90 minute) incubation of CSC with high concentrations of the poorly hydrolyzable ATP analog ATP-γ-S or the non-hydrolyzable analog AppNHp prior to triggering has no effect on the Ca^{2+} sensitivity or extent of fusion, suggesting that this possibility is unlikely. The possible presence of a tightly bound, distantly related ATPase such as a homolog of p97 (Peters et al., 1990) has not been definitively ruled out, since we have found no antibody to this protein that specifically detects a sea urchin homolog. However, our data showing that exocytosis is insensitive to the presence of ATP and non-hydrolyzable analogs of ATP clearly suggest that it is unlikely that ATPase activity of such a protein is required for CV membrane fusion.

A third indication that the CV protein target of thiol reagents is not NSF or another membrane-associated ATPase, is based on the observation that soluble cytosolic proteins can restore fusion activity to NEM-inactivated fusion events in which NSF participates (Rothman, 1994) because of an exchange of membrane-bound and soluble NSF. However, sea urchin cytosol cannot restore fusion activity to NEM-treated CSC (Whalley and Sokoloff, 1994; Zimmerberg et al., 1996), despite our current finding that NSF is present in this cytosol. It is known that high concentrations of NEM alkylate SNARE proteins, as well as NSF (Meffert et al., 1996), and it has been demonstrated that cysteine residues of SNAP-25 are required both for SNARE complex disassembly and exocytosis in PC12 cells (Washbourne et al., 2001). Other proteins involved in membrane fusion might also contain important cysteine residues that would be targets for such inhibitors. Here we have shown that partial inhibition of exocytosis, using a lower concentration of NEM or using a more hydrophilic thiol-reactive agent of high molecular mass (D10PDP) and a charged NEM derivative (AMSDS), which were expected to react with fewer proteins, also resulted in inhibition of exocytosis. This inhibition of exocytosis was not reversible with the addition of

cytosol. We do not know the identity of the protein(s) that are targets for the thiol-reactive inhibitors that we used but it is unlikely that they are SNARE proteins alone, as previous studies of CV fusion have shown that SNAREs are not essential for fusion, although they might play regulatory functions (Coorssen et al., 1998; Coorssen et al., 2003; Szule et al., 2003).

Fourthly, an analysis of the amino acid sequence of sea urchin NSF presented in this paper indicates that it lacks the cysteine residue (Cys264) that is thought to be the target for alkylation of mammalian NSF (Tagaya et al., 1993). This finding is similar to that for the yeast homolog of NSF (Sec18) where a threonine/cysteine substitution is thought to render Sec18 much less sensitive to NEM compared to mammalian NSF (Wilson et al., 1989; Haas and Wickner, 1996). A further analysis of Sec18 (Steel et al., 1999) has shown that while its ATPase activity is insensitive to NEM up to concentrations of 10 mM, there is nevertheless an inhibitory effect of this treatment on in vitro Golgi fusion (Wilson et al., 1989). This suggests that a cysteine distinct from that of the D1 loop of the ATP binding pocket is important in some physiological aspect of NSF/Sec18 function.

We wanted to know if NSF was capable of binding to sea urchin egg exocytotic membrane and assessed this using the ATP-dependent binding of NSF to membranes containing SNARE proteins (Wilson et al., 1992). Binding of egg cytosolic NSF to CV in vitro was demonstrated (Fig. 5A), with 11 hexamers of NSF bound per CV in the presence of ATP-γ-S. These data represent a lower limit for the number of NSF binding sites in the CV membrane, since no attempt to determine maximum binding was made. Nevertheless, the binding data are consistent with the documented presence in CV membranes of the SNARE proteins syntaxin, SNAP-25, and VAMP (Coorssen et al., 1998; Tahara et al., 1998; Bi et al., 1995; Avery et al., 1997; Conner et al., 1997) or other as yet unidentified NSF binding partners. Perhaps most consistent with a role for NSF in the recycling of membrane proteins after a round of membrane fusion is its very high plasma membrane density, even on membranes denuded of CV and ER. Apparently endogenous NSF is localized adjacent to or between native CV docking sites, making it ideally placed for participation during or immediately after endocytosis (Whalley et al., 1995). It has been shown that there is an alteration in the membrane fusion properties of early endosomal membranes following exocytosis in sea urchin eggs (Ikebuchi et al., 2001). This might reflect a rearrangement of SNARE proteins in the endosome membrane catalyzed by NSF. However, since the binding sites of NSF have not been positively identified in our experiments, among other reasons, these data do not necessarily imply a role for SNARE proteins in CV membrane fusion.

We developed an antibody against the D1-D2 ATPase binding domain boundary (Whiteheart et al., 1994) of sea urchin NSF and used this to probe the possible involvement of NSF in sea urchin egg CV fusion. The antibody was able to bind to NSF from cytosol and was functionally inhibitory as it prevented the ATP-γ-S-dependent association of NSF with exocytotic membranes (Fig. 5B). As the antibody was functionally inhibitory, we used it to determine if there was a functional role for NSF during exocytosis. We found that the antibody had no effect on the Ca^{2+} sensitivity of exocytosis (Fig. 6). One caveat with such a result is that antibody accessibility to the fusion site might be limited because of the close contact of CV and PM (Vacquier, 1975; Chandler and Heuser, 1979). In order to rule out this as an issue, we also treated isolated CVs with an antibody before determining the Ca^{2+} sensitivity of CV-CV fusion. Again, the anti-NSF antibody was without effect suggesting that NSF is not required for CV fusion (Fig. 6).

We used our NSF binding assay to further assess any potential role for NSF during CV fusion. We tested the NEM sensitivity of NSF binding and, at the same time tested the sensitivity of CV exocytosis towards NEM. As can be seen in Fig. 7, exocytosis and NSF are differentially sensitive to inhibition by NEM. There is a clear difference between the sensitivity of CV exocytosis and NSF binding to inhibition by NEM. There is no effect on the binding of NSF to cortical membranes except at 20 mM NEM, the highest concentration tested. In contrast, inhibition of CV exocytosis starts with 0.5 mM NEM, and is almost completely inhibited by 5 mM NEM. These data suggest that the protein target for NEM that inhibits CV exocytosis is distinct from NSF.

Despite this evidence, it still seemed possible that a few NSF protein molecules might be tightly bound to the egg cortex and might be inaccessible to externally supplied nucleotides and unable to exchange with cytosolic NSF. In addition of course, it is possible that the role of NSF in fusion might be independent of its ATPase activity (Muller et al., 1999). We analyzed whether NSF was present in the isolated exocytotic apparatus using our NSF. We documented the presence of ~8 hexamers of NSF in the planar cortex per square micron of cell surface complex. However, these copies of NSF were still present on plasma membranes prepared from IPCs by mechanical removal of most of the CVs, indicating that NSF is not associated with CVs that contain the minimally essential components for triggered fusion. This suggests that NSF in the egg cortex is not bound to transmembrane 'SNAREpin' complexes (Weber et al., 1998) or any other inter-membrane complexes that involve CV membrane proteins, unless these complexes had been torn out of the vesicle membrane by the mechanical force used to purify the CVs and remained attached preferentially to the plasma membrane. This is unlikely since CV membranes are not disrupted upon removal from the PM – the CVs remain intact and are fully capable of Ca^{2+}-dependent fusion.

In contrast to data obtained with the isolated planar cortex, the NSF content in isolated CVs was always less than one hexamer per CV (Fig. 8). Despite this, every CV is fusion-competent, as demonstrated in both homotypic fusion assays (Coorssen et al., 1998; Tahara et al., 1998) and after reconstitution with the plasma membrane (Crabb and Jackson, 1985; Whalley and Whitaker, 1988). This argues strongly that NSF is not an obligatory part of the 'fusion machine', of which there are ~8-10 separate functional units per CV (Coorssen et al., 1998; Vogel et al., 1996) as compared to a mean of 0.17 NSF hexamers per CV. These findings contrast with reports that NSF is associated with synaptic vesicles (Hong et al., 1994) and clathrin-coated vesicles (Steel et al., 1996) that are isolated from a state that precedes docking. In addition, it is possible that the low levels of NSF found in CV preparations is the result of a small amount of contaminating plasma membrane. This cannot be evaluated biochemically at the

present time, because of a lack of suitable antibodies for plasma membrane marker proteins. Microscopic inspection of CV preparations shows that there are no large contaminating pieces of plasma membrane, although the presence of small pieces attached to CVs cannot be evaluated using this criterion. However, if even small fragments of PM were associated with CVs we would expect that the addition of Ca^{2+} would lead to membrane fusion in suspensions of CVs, a loss of CV membrane integrity and the release of CV contents. This was never observed.

The data presented in this paper indicate that NSF need not be associated with Ca^{2+}-dependent fusion-competent CV membranes, either in isolation or in isolated planar cortices in which the vesicles are already docked and are primed for fusion. Therefore, NSF is unlikely to play any role in the final stages of membrane fusion in this biological system. Any role that NSF might play in promoting sea urchin egg CV exocytosis must have been performed before the CV reached a docked and primed state. More likely, the NSF bound to the cytosolic surface of the egg may reflect a masking of cis-SNARE complexes during the metabolically quiescent period prior to fertilization. This masking might serve to prevent precocious docking of vesicles and premature establishment of embryonic polarity, since the targeting of specific vesicles to specific locations seems to be mediated, in part, by cognate SNARE recognition (McNew et al., 2000; Pelham, 2001).

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References

Avery, J., Hodel, A. and Whitaker, M. (1997). In vitro exocytosis in sea urchin eggs requires a synaptobrevin-related protein. *J. Cell Sci.* **110**, 1555-1561.

Baker, P. F. and Whitaker, M. (1978). Influence of ATP and calcium on the cortical reaction in sea urchin eggs. *Nature* **276**, 513-515.

Banerjee, A., Barry, V. A., DasGupta, B. R. and Martin, T. F. J. (1996). N-Ethylmaleimide-sensitive factor acts at a prefusion ATP-dependent step in Ca^{2+}-activated exocytosis. *J. Biol. Chem.* **271**, 20223-20226.

Bi, G.-Q., Alderton, J. M. and Steinhardt, R. A. (1995). Calcium-regulated exocytosis is required for cell membrane resealing. *J. Cell Biol*. **131**, 1747-1758.

Blank, P. S., Cho, M.-S., Vogel, S. S., Kaplan, D., Kang, A., Malley., J. and Zimmerberg, J. (1998). Submaximal responses in calcium-triggered exocytosis are explained by differences in the calcium sensitivity of individual secretory vesicles. *J. Gen. Physiol.* **112**, 559-567.

Block, M. R., Glick, B. S., Wilcox, C. A., Wieland, F. T. and Rothman, J. E. (1988). Purification of an N-ethylmaleimide-sensitive protein catalyzing vesicular transport. *Proc. Natl. Acad. Sci. USA* **85**, 7852-7856.

Brugger, B., Nickel, W., Weber, T., Parlati, F., McNew, J. A., Rothman, J. E. and Sollner, T. (2000). Putative fusogenic activity of NSF is restricted to a lipid mixture whose coalescence is also triggered by other factors. *EMBO J*. **19**, 1272-1278.

Brunger, A. T. (2001). Structural insights into the molecular mechanism of calcium-dependent vesicle-membrane fusion. *Curr. Opin. Struct. Biol.* **11**, 163-173.

Chandler, D. E. (1984). Comparison of quick-frozen and chemically fixed sea-urchin eggs: structural evidence that cortical granule exocytosis is preceded by a local increase in membrane mobility. *J. Cell Sci.* **72**, 23-36.

Chandler, D. E. (1991). Multiple intracellular signals coordinate structural dynamics in the sea urchin egg cortex at fertilization. *J. Elec. Micro. Tech.* **17**, 266-293.

Chandler, D. E. and Heuser, J. (1979). Membrane fusion during secretion: cortical granule exocytosis in sea urchin eggs as studied by quick-freezing and freeze-fracture. *J. Cell Biol.* **83**, 91-108.

Clary, D. O. and Rothman, J. E. (1990). Purification of three related peripheral membrane proteins needed for vesicular transport. *J. Biol. Chem.* **265**, 10109-10117.

Clary, D. O., Griff, I. C. and Rothman, J. E. (1990). SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. *Cell* **61**, 709-721.

Conner, S., Leaf, D. and Wessel, G. (1997). Members of the SNARE hypothesis are associated with cortical granule exocytosis in the sea urchin egg. *Mol. Reprod. Dev*. **48**, 106-118.

Coorssen, J. R., Blank, P. S., Tahara, M. and Zimmerberg, J. (1998). Biochemical and functional studies of cortical vesicle fusion: the SNARE complex and Ca^{2+} sensitivity. *J. Cell Biol*. **143**, 1845-1857.

Coorssen, J. R., Blank, P. S., Albertorio, F., Bezrukov, L., Kolosova, I., Chen, X., Backlund, P. S. Jr and Zimmerberg, J. (2003). Regulated secretion: SNARE density, vesicle fusion and calcium dependence. *J. Cell Sci.* **116**, 2087-2097.

Crabb, J. H. and Jackson, R. C. (1985). In vitro reconstitution of exocytosis from plasma membrane and isolated secretory vesicles. *J. Cell Biol*. **101**, 2263-2273.

Glick, B. S. and Rothman, J. E. (1987). Possible role for fatty acyl-coenzyme A in intracellular protein transport. *Nature* **326**, 309-312.

Haggerty, J. G. and Jackson, R. C. (1983). Release of granule contents from sea urchin egg cortices. New assay procedures and inhibition by sulfhydryl-modifying reagents. *J. Biol. Chem*. **258**, 1819-1825.

Hay, J. C. and Martin, T. F. J. (1992). Resolution of regulated secretion into sequential Mg-ATP-dependent and calcium-dependent stages mediated by distinct cytosolic proteins. *J. Cell Biol*. **119**, 139-151.

Haas, A. and Wickner, W. (1996). Homotypic vacuole fusion requires Sec17p (yeast alpha-SNAP) and Sec18p (yeast NSF). *EMBO J.* **15**, 3296-3305.

Heidelberger, R. (1998). Adenosine triphosphate and the late steps in calcium-dependent exocytosis at a ribbon synapse. *J. Gen. Physiol.* **111**, 225-241.

Holz, R. W., Bittner, M. A., Peppers, S. C., Senter, R. A. and Eberhard, D. A. (1989). MgATP-independent and MgATP-dependent exocytosis. *J. Biol. Chem.* **264**, 5412-5419.

Hong, R.-M., Mori, H., Fukui, T., Moriyami, Y., Futai, M., Yamamoto, A., Tashiro, Y. and Tagaya, M. (1994). Association of N-ethylmaleimide-sensitive factor with synaptic vesicles. *FEBS Lett*. **350**, 253-257.

Howell, T. W., Cockcroft, S. and Gomperts, B. D. (1987). Essential synergy between Ca^{2+} and guanine nucleotides in exocytotic secretion from permeabilized mast cells. *J. Cell Biol*. **105**, 191-197.

Ikebuchi, Y., Baibakov, B., Smith, R. M. and Vogel, S. S. (2001). Plasma membrane resident ‘fusion complexes’ mediate reconstituted exocytosis. *Traffic* **2**, 654-667.

Jackson, R. C. and Modern, P. A. (1990). N-ethylmaleimide-sensitive protein(s) involved in cortical exocytosis in the sea urchin egg: localization to both cortical vesicles and plasma membrane. *J. Cell Sci*. **96**, 313-321.

Laidlaw, M. and Wessel, G. M. (1994). Cortical granule biogenesis is active throughout oogenesis in sea urchins. *Development* **120**, 1325-1333.

Lin, R. C. and Scheller, R. H. (2000). Mechanisms of synaptic vesicle exocytosis. *Ann. Rev. Cell Dev. Biol*. **16**, 19-49.

McNew, J. A., Parlati, F., Fukuda, R., Johnston, R. J., Paz, K., Paumet, F., Sollner, T. H. and Rothman, J. E. (2000). Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature* **407**, 153-159.

Mayer, A. and Wickner, W. (1997). Docking of yeast vacuoles is catalyzed by the Ras-like GTPase Ypt7p after symmetric priming by Sec18p (NSF). *J. Cell Biol.* **136**, 307-317.

Mayer, A., Wickner, W. and Haas, A. (1996). Sec18p (NSF)-driven release of Sec17p (alpha-SNAP) can precede docking and fusion of yeast vacuoles. *Cell* **85**, 83-94.

Meffert, M. K., Calakos, N. C., Scheller, R. H. and Schulman, H. (1996). Nitric oxide modulates synaptic vesicle docking fusion reactions. *Neuron* **16**, 1229-1236.

Morgan, A. and Burgoyne, R. D. (1995). Is NSF a fusion protein? *Trends Cell Biol*. **5**, 335-339.

Moy, G. W., Kopf, G. S., Gache, C. and Vacquier, V. D. (1983). Calcium-mediated release of glucanase activity from cortical granules of sea urchin eggs. *Dev. Biol*. **100**, 267-274.

Muller, J. M., Rabouille, C., Newman, R., Shorter, J., Freemont, P., Schiavo, G., Warren, G. and Shima, D. T. (1999). An NSF function distinct from ATPase-dependent SNARE disassembly is essential for Golgi membrane fusion. *Nat. Cell Biol*. **1**, 335-334.

Otter-Nilsson, M., Hendriks, R., Pecheur-Huet, E-I., Hoekstra, D. and Nilsson, T. (1999). Cytosolic ATPases, p97 and NSF, are sufficient to mediate rapid membrane fusion. *EMBO J.* **18**, 2074-2083.

Parsons, T. D., Coorssen, J. R., Horstmann, H. and Almers, W. (1995). Docked granules, the exocytic burst, and the need for ATP hydrolysis in endocrine cells. *Neuron* **15**, 1085-1096.

Pelham, H. R. (2001). SNAREs and the specificity of membrane fusion. *Trends Cell Biol.* **11**, 99-101.

Peres, A. and Bernardini, G. (1985). The effective membrane capacitance of Xenopus eggs: its relations with membrane conductance and cortical granule exocytosis. *Pfugers Arch*. **404**, 266-272.

Peters, J.-M., Walsh, M. J. and Franke, W. W. (1990). An abundant and ubiquitous homo-oligomeric ring-shaped ATPase particle related to the putative vesicle fusion proteins Sec18p and NSF. *EMBO J.* **9**, 1757-1767.

Peters, C., Bayer, M. J., Buhler, S., Andersen, J. S., Mann, M. and Mayer, A. (2001). Trans-complex formation by proteolipid channels in the terminal phase of membrane fusion. *Nature* **409**, 581-588.

Rothman, J. E. (1994). Mechanisms of intracellular protein transport. *Nature* **372**, 55-63.

Sardet, C. (1984). The ultrastructure of the sea urchin egg cortex before and after fertilization. *Dev. Biol.* **105**, 196-297.

Sasaki, H. and Epel, D. (1983). Cortical vesicle exocytosis in isolated cortices of sea urchin eggs: description of a turbidometric assay and its utilization in studying effects of different media on discharge. *Dev. Biol*. **98**, 327-337.

Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J. E. (1993). SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362**, 318-324.

Steel, G. J., Tagaya, M. and Woodman, P. G. (1996). Association of the fusion protein NSF with clathrin-coated vesicle membranes. *EMBO J*. **15**, 745-752.

Steel, G. J., Laude, A. J., Boojawan, A., Harvey, D. J. and Morgan, A. (1999). Biochemical analysis of the Saccharomyces cerevisiae SEC18 gene product: implications for the molecular mechanism of membrane fusion. *Biochemistry* **38**, 7764-7772.

Swann, K. and Whitaker, M. J. (1986). The part played by inositol trisphosphate and calcium in the propagation of the fertilization wave in sea urchin eggs. *J. Cell Biol*. **103**, 2333-2342.

Szule, J. A., Jarvis, S. E., Hibbert, J. E., Spafford, J. D., Braun, J. E. A., Zamponi, G. W., Wessel, G. M. and Coorssen, J. R. (2003). Calcium-triggered membrane fusion proceeds independently of specific presynaptic proteins. *J. Biol. Chem.* **278**, 24251-24254.

Tagaya, M., Wilson, D. W., Brunner, M., Arango, N. and Rothman, J. E. (1993). Domain structure of an N-ethylmaleimide-sensitive fusion protein involved in vesicular transport. *J. Biol. Chem*. **268**, 2662-2666.

Tahara, M., Tasaka, K., Masumoto, N., Mammoto, A., Ikebuchi, Y. and Miyake, A. (1996). Dynamics of cortical granule exocytosis at fertilization in living mouse eggs. *Am. J. Physiol*. **270**, C1354-C1361.

Tahara, M., Coorssen, J. R., Timmers, K., Blank, P. S., Whalley, T., Scheller, R. and Zimmerberg, J. (1998). Calcium can disrupt the SNARE protein complex on sea urchin egg secretory vesicles without irreversibly blocking fusion. *J. Biol. Chem*. **273**, 33667-33673.

Terasaki, M., Henson, J., Begg, D., Kaminer, B. and Sardet, C. (1991). Characterization of sea urchin egg endoplasmic reticulum in cortical preparations. *Dev. Biol*. **148**, 398-401.

Ungermann, C., Nichols, B. J., Pelham, H. R. and Wickner, W. (1998a). A vacuolar v-t-SNARE complex, the predominant form *in vivo* and on isolated vacuoles, is disassembled and activated for docking and fusion. *J. Cell Biol*. **140**, 61-69.

Ungermann, C., Sato, K. and Wickner, W. (1998b). Defining the functions of trans-SNARE pairs. *Nature* **396**, 543-548.

Vacquier, V. D. (1975). The isolation of intact cortical granules from sea urchin eggs: calcium ions trigger granule discharge. *Dev. Biol*. **43**, 62-74.

Vogel, S. S. and Zimmerberg, J. (1992). Proteins on exocytic vesicles mediate calcium-triggered fusion. *Proc. Natl. Acad. Sci. USA* **89**, 4749-4753.

Vogel, S. S., Chernomordik, L. V. and Zimmerberg, J. (1992). Calcium-triggered fusion of exocytotic granules requires proteins in only one membrane. *J. Biol. Chem*. **267**, 25640-25643.

Vogel, S. S., Blank, P. S. and Zimmerberg, J. (1996). Poisson-distributed active fusion complexes underlie the control of the rate and extent of exocytosis by calcium. *J. Cell Biol*. **134**, 329-338.

Walker, J. E., Saraste, M., Runswick, M. J. and Gay, N. J. (1982). Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J*. **1**, 945-951.

Washbourne, P., Cansino, V., Mathews, J. R., Graham, M., Burgoyne, R. D. and Wilson, M. C. (2001). Cysteine residues of SNAP-25 are required for SNARE disassembly and exocytosis but not for membrane targeting. *Biochem. J.* **357**, 625-634.

Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachi, M., Parlati, F., Sollner, T. and Rothman, J. E. (1998). SNAREpins: minimal machinery for membrane fusion. *Cell* **92**, 759-772.

Whalley, T. and Whitaker, M. (1988). Exocytosis reconstituted from the sea urchin egg is unaffected by calcium pretreatment of granules and plasma membrane. *Biosci. Rep*. **8**, 335-343.

Whalley, T. and Sokoloff, A. (1994). The N-ethylmaleimide-sensitive protein thiol groups necessary for sea urchin egg cortical granule exocytosis are highly exposed to the medium and are required for triggering by Ca^{2+}. *Biochem. J*. **302**, 391-396.

Whalley, T., Crossley, I. and Whitaker, M. (1991). Phosphoprotein inhibition of calcium-stimulated exocytosis in sea urchin eggs. *J. Cell Biol.* **113**, 769-778.

Whalley, T., Terasaki, M., Cho, M.-S. and Vogel, S. S. (1995). Direct membrane retrieval into large vesicles following exocytosis in sea urchin eggs. *J. Cell Biol*. **131**, 1183-1192.

Whitaker, M. (1987). How calcium may cause exocytosis in sea urchin eggs. *Biosci. Rep*. **7**, 383-397.

Whiteheart, S. W., Brunner, M., Wilson, D. W., Wiedmann, M. and Rothman, J. E. (1992). Soluble N-ethylmaleimide-sensitive fusion attachment proteins (SNAPs) bind to a multi-SNAP receptor complex in Golgi membranes. *J. Biol. Chem*. **267**, 12239-12243.

Whiteheart, S. W., Rossnagel, K., Buhrow, S. A., Brunner, M., Jaenicke, R. and Rothman, J. E. (1994). N-ethylmaleimide-sensitive fusion protein: a trimeric ATPase whose hydrolysis of ATP is required for membrane fusion. *J. Cell Biol*. **126**, 945-954.

Wilson, D. W., Wilcox, C. A., Flynn, G. C., Chen, E., Kuang, W. J., Henzel, W. J., Block, M. R., Ullrich, A. and Rothman, J. E. (1989). A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature* **339**, 355-359.

Wilson, D. W., Whiteheart, S. W., Wiedmann, M., Brunner, M. and Rothman, J. E. (1992). A multisubunit particle implicated in membrane fusion. *J. Cell Biol*. **117**, 531-538.

Xu, Z. Y., Mayer, A., Muller, E. and Wickner, W. (1997). A heterodimer of thioredoxin and I(B)2 cooperates with Sec18p (NSF) to promote yeast vacuole inheritance. *J. Cell Biol*. **136**, 299-306.

Zimmerberg, J. and Liu, J. (1988). Ionic and permeability requirements for exocytosis in vitro in sea urchin eggs. *J. Membr. Biol*. **101**, 199-207.

Zimmerberg, J., Sardet, C. and Epel, D. (1985). Exocytosis of sea urchin egg cortical vesicles in vitro is retarded by hyperosmotic sucrose: kinetics of fusion monitored by quantitative light-scattering microscopy. *J. Cell Biol*. **101**, 2398-2410.

Zimmerberg, J., Vogel, S. S., Whalley, T., Plonsky, I., Sokoloff, A., Chanturia, A. and Chernomordik, L. V. (1996). Intermediates in membrane fusion. *Cold Spring Harbor Symp. Quant. Biol*. **60**, 589-599.