In vitro exocytosis in sea urchin eggs requires a synaptobrevin-related protein

Julia Avery*, Alois Hodel and Michael Whitaker†

Department of Physiological Sciences, Medical School, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK

*Author for correspondence (e-mail: michael.whitaker@ncl.ac.uk)
†Present address: Department of Pharmacology, Yale University School of Medicine, Yale University, New Haven, Connecticut 06510, USA

SUMMARY

Sea urchin eggs provide an efficient in vitro model of exocytosis. We have identified proteins in sea urchin eggs that cross-react with antibodies to mammalian synaptobrevin, synaptotagmin, SNAP-25, syntaxin and rab3a. We show that these proteins are localized to the sea urchin egg cortex, using western blotting and immunocytochemistry. Tetanus toxin light chain cleaves the synaptobrevin-related protein in vitro and inhibits calcium-induced exocytosis. These data demonstrate a conservation between phyla of protein sequence and molecular mechanisms thought to facilitate exocytosis and show that the sea urchin egg provides a unique in vitro exocytotic model with which to study the conserved protein machinery of membrane fusion during secretion.

Key words: Synaptobrevin, Tetanus toxin, Sea urchin egg

INTRODUCTION

Although the mechanism of exocytosis remains unclear, rapid progress has recently been made in identifying proteins that are involved in membrane fusion. Much of this progress has been achieved by studying neuronal exocytosis. According to the current concept, a 7 S protein complex containing the proteins synaptobrevin, syntaxin, synaptotagmin and SNAP-25 mediates docking and fusion of synaptic vesicles to the plasma membrane of the nerve terminal (Bennett et al., 1992; Söllner et al., 1993). The components of the 7 S complex are highly conserved throughout evolution and have been identified in a wide variety of secretory cells (Ferro-Novick and Jahn, 1994; Roth and Burgoyne, 1994; Hodel et al., 1994; Jacobsson et al., 1994; Regazzi et al., 1995). Compelling evidence that the component proteins are essential for secretion has been obtained by the use of bacterial neurotoxins that selectively cleave either synaptobrevin, syntaxin, or SNAP-25 and concomitantly inhibit exocytosis (Schiavo et al., 1992, 1993; Link et al., 1992; Blasi et al., 1993a,b; Binz et al., 1994). Another line of evidence that implicates these proteins in exocytosis is their identification as the membrane receptors (SNAREs) for the general fusion proteins NSF (N-ethylmaleimide-sensitive factor) and SNAPs (soluble NSF-attachment proteins) (Söllner et al., 1993). NSF and SNAPs were originally identified as soluble factors essential for membrane trafficking in cell free extracts (for review see Rothman and Orci, 1992). Studies of the neuronal fusion proteins in detergent extracts show that the 7 S protein complex binds NSF and SNAPs to form a 20 S putative fusion complex (Söllner et al., 1993). The complex disassembles in vitro upon ATP hydrolysis by NSF, an event which was proposed to be the driving force for membrane fusion (Söllner et al., 1993). Rab3a, a low molecular mass GTP-binding protein, was proposed to regulate assembly of the 7 S fusion complex (Rothman, 1994).

Sea urchin eggs undergo a regulated, calcium-dependent exocytosis of their cortical secretory granules at fertilization (Whitaker, 1994). The protein-containing secretory granules are around 1 μm in diameter and are docked to the plasma membrane in the mature oocyte (Schuel, 1978). A pure preparation of plasma membrane and attached cortical granules can be obtained by sticking eggs to a polylysine-coated surface and breaking them open by vigorous washing to leave the egg cortex (plasma membrane and granules) attached to the substrate (Vacquier, 1975). When physiological levels of calcium are added to the exposed cytoplasmic face of the plasma membrane, all the secretory granules fuse with the plasma membrane (Vacquier, 1975; Baker and Whitaker, 1978; Moy et al., 1983; Crabb et al., 1987). No other soluble proteins or cofactors are required.

The sea urchin egg provides the only available example of efficient, calcium-mediated exocytosis in vitro, perhaps because in the sea urchin egg, docking is complete and soluble components unnecessary. Earlier reports showed that membrane resealing, a process thought to be based upon exocytosis, is inhibited by microinjecting neurotoxins, suggesting that proteins homologous to the components of the 7 S complex are present in sea urchin eggs (Steinhardt et al., 1991; Bi et al., 1995). Our aim was to determine whether these proteins are present in the sea urchin egg and are involved in cortical granule exocytosis. We find evidence for the presence of homologues by western blotting and immunocytochemistry and...
show that docked granules are sensitive to tetanus toxin light chain (TeTx LC). The in vitro exocytotic system of the sea urchin egg cortex thus offers a useful and uniquely simple model for determining how proteins of the 7 S complex bring about docking and fusion of secretory granules with the plasma membrane.

**MATERIALS AND METHODS**

**Gamete handling**

Sea urchins of the species *Lytechinus pictus* (Pacific Biomarine Laboratories Inc., Venice, CA, USA and Marinus Inc., Long Beach CA, USA) were held in thermostatted tanks containing natural seawater at 15-16°C in order to keep them continually gravid. They were fed fortnightly on seaweed (Marine Biological Station, Millport, Scotland). Eggs were obtained by the intracoelomic injection of female urchins with 100 μl 0.5 M KCl and were collected by using inverted urchins in a beaker of artificial seawater (435 mM NaCl, 40 mM MgCl₂, 15 mM MgSO₄, 11 mM CaCl₂, 10 mM KCl, 2.5 mM NaHCO₃, 1 mM EDTA, pH 8.0).

**Investigating cortical granule exocytosis in vitro**

Egg jelly was removed by multiple passages through a Nitex mesh and cortical granules were prepared as previously described (Whalley and Whitaker, 1988). Briefly, eggs were attached to poly(L-lysine)-coated coverslips and were rinsed gently with intracellular medium: IM (220 mM potassium glutamate, 500 mM glycine, 10 mM NaCl, 5 mM MgCl₂, 2.5 mM ATP, 2 mM EGTA, pH 6.7). The eggs were sheared with a jet of medium, leaving isolated cortical granules attached to the glass. Exocytosis in response to calcium was assessed by counting the number of granules that fused after calcium addition, using phase contrast microscopy. Calcium-EGTA buffers were prepared using the constants of Martell and Sillen (1964).

**Subcellular fractionation and protein preparation**

Cortex, plasma membrane and cortical granule protein fractions were prepared as described previously (Whalley and Whitaker, 1988; Crabb and Jackson, 1985) in IM containing 1 μg/ml leupeptin, 1 μg/ml pepstatin and 0.1 mM PMSF. To obtain whole egg membrane and cytosolic protein fractions, eggs were lysed in the same buffer and centrifuged at 100,000 g for 1 hour at 4°C in a Sorvall ultracentrifuge. Proteins from all samples were extracted using chloroform/methanol (2:1, v/v). The precipitated protein was washed twice with distilled water before dissolution in SDS-PAGE sample buffer. Protein content was determined using the bicinchoninic acid assay protocol (Pierce Immunochemicals Ltd, Illinois, USA) using bovine serum albumin as a standard.

**SDS-PAGE and western blot analysis**

SDS-PAGE (Laemmli, 1970) was performed on 12% (w/v) resolving gels using a vertical minigel apparatus at 175 V. Proteins were transferred from 12% SDS-PAGE gels to polyvinylidene difluoride transfer membranes (PVDF, Millipore Corp., MA, USA) according to the method of Towbin (1979) at a constant voltage of 5 V for 1 hour. After blocking in PBS (137 mM NaCl, 2.7 mM KCl, 7.0 mM Na₂HPO₄, 1.25 mM NaH₂PO₄, 1.5 mM KH₂PO₄) containing 0.01% Tween-20, 5% milk protein and 1.5% bovine serum albumin, the membranes were incubated with primary antibody diluted 1:500 in PBS containing 0.01% Tween-20, 5% goat serum and 1.5% bovine serum albumin. After washing, blots were incubated with 1:5,000 dilution horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Pierce Immunochemicals Ltd, Illinois, USA) in the same buffer. Immunoreactive proteins were visualized using the Enhanced Chemiluminescence System (Amersham International plc), according to the manufacturer’s instructions. The primary antibodies used for western blotting were as follows: anti-synaptobrevin monoclonal antibody (C1 10.1) (Baumert et al., 1989), anti-rab3 monoclonal antibody (C1 42.1) (Matteoli et al., 1991), anti-synaptotagmin polyclonal antipeptide antibody (Takahashi et al., 1991), anti-syntaxin monoclonal antibody (6D2) and anti- SNAP-25 polyclonal antibody (Yoshida et al., 1992).

**Immunofluorescence and confocal laser scanning microscopy**

Cortices were prepared on coverslips, as described above, before fixing in cortex isolation buffer (0.6 M mannitol, 2.5 mM MgCl₂, 20 mM EGTA, 50 mM Hepes, 50 mM Pipes, pH 6.8) containing 3% formaldehyde and 0.5% gluteraldehyde for 30 minutes at room temperature. Fixed cortices were subsequently incubated in aldehyde quench solution (75 mM KCl, 2 mM MgCl₂, 10 mM EGTA, 150 mM glycine, 320 mM sucrose, 2.5 mM Pipes, pH 6.8) for 1 hour at 4°C. Cortices were incubated with primary antibody diluted 1:50 in PBS containing 1% bovine serum albumin, 2% goat serum and 0.1% Triton-X-100, pH 7.5, followed by incubation with fluorescein-conjugated goat anti-mouse IgG (Pierce Immunochemicals Ltd, Illinois, USA) diluted 1:5,000 in the same buffer and were then mounted with glycerol on glass slides. Cortices were stained with 1.1-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiIC₁₈) according to the method of Terasaki et al. (1991). Conventional light microscopy was performed using a Nikon optiphot microscope and confocal microscopy was performed on the Leica laser scanning microscope.

**RESULTS**

**Homologues of the neuronal fusion proteins are present in sea urchin eggs**

We examined the expression of homologues of the putative neuronal exocytotic fusion proteins synaptobrevin, syntaxin, SNAP-25, synaptotagmin and rab3a in *Lytechinus pictus* eggs. Western blot analysis of subcellular protein fractions revealed the expression of several homologues, as judged by immunoreactivity with antibodies to mammalian proteins (Fig. 1). A polyclonal antibody against a conserved sequence within the first C2 domain of synaptotagmin recognized a protein of approximately 65 kDa (Fig. 1A). A monoclonal antibody against rab3a (C1 42.1) recognized a protein of approximately 27 kDa (Fig. 1A) and a monoclonal antibody against synaptobrevin (C1 10.1) identified a protein of approximately 17.5 kDa (Fig. 1A). All three of the immunogenic proteins were specifically localized to the egg cortex and none were detected in the cytosol, consistent with the localization of the corresponding neuronal proteins. A monoclonal antibody against syntaxin (6D2) identified a protein of approximately 35 kDa (Fig. 1B) and a polyclonal antibody against rat brain SNAP-25 recognized a protein of approximately 25 kDa (Fig. 1B). The immunoreactivity found appeared, in all but the case of syntaxin, to be monospecific and was not due to unspecific binding by the secondary antibody (Fig. 1B). In addition, the relative molecular masses of the immunoreactive proteins correspond well with those of their mammalian counterparts (Baumert et al., 1989; Oyler et al., 1989; Inoue et al., 1992), although the putative sea urchin homologues of synaptotagmin and synaptobrevin run at slightly lower molecular masses (Fig. 1A). This suggests the presence of bona fide sea urchin homologues for synaptobrevin, syntaxin, SNAP-25, synaptotagmin and rab3a in the unfertilized egg.

**Localization of synaptobrevin immunoreactivity to the cortical granule membrane**

The localization of the 17.5 kDa protein recognized by mono-
clonal antibody C1 10.1 was further investigated by using the antibody to label the isolated egg cortex by indirect immunofluorescence. Optical sections parallel and perpendicular to the plane of the plasma membrane were obtained using confocal microscopy (Fig. 2A,B). The ‘honeycomb’ pattern of staining obtained is consistent with staining of the layer of cortical granules and suggests that the immunoreactive protein is localized to the cortical granule membrane. Staining was uniform throughout the cortex, suggesting that the 17.5 kDa protein is evenly distributed throughout the granule population. No staining of the plasma membrane was observed. In the control, in which cortices were incubated with the fluorescein-conjugated secondary antibody only, no fluorescence was seen (Fig. 2A, inset). In order to confirm this interpretation, the pattern of staining was compared to the pattern of staining obtained with the lipophilic carbocyanine dye, DiIC18. We found, as Terasaki (1991) reported, that DiIC18 stains plasma membrane, endoplasmic reticulum and, occasionally, cortical granules in the cortex (Fig. 2C,D). However, the staining pattern is clearly different from the one obtained using the anti-synaptobrevin antibody. From this, we conclude that anti-synaptobrevin immunoreactivity is found only at the periphery of cortical granules.

Tetanus toxin light chain cleaves the synaptobrevin-like protein in vitro

Tetanus toxin light chain (TeTx LC) is a zinc-dependent endoprotease that specifically cleaves members of the synaptobrevin protein family (Schiavo et al., 1992; Link et al., 1992). Therefore, structural similarities between the sea urchin 17.5 kDa protein and synaptobrevin would be demonstrated if it was similarly cleaved by TeTx LC. When a detergent-extracted membrane protein fraction was incubated with TeTx LC at 37°C, subsequent immunoblotting revealed the 17.5 kDa protein, anti-synaptobrevin, anti-rab3, and anti-syntaxin. The antibody used in B differed in that it gave increased background staining. Bound antibodies were visualized using the enhanced chemiluminescence system (ECL, Amersham Int. plc).

Fig. 1. Immunoblotting of egg subcellular protein fractions. Sea urchin egg cortical and cytosolic protein fractions and rat brain homogenate (50 µg/lane) were separated by SDS-PAGE, electrotransferred onto PVDF membranes and immunostained with antibodies against synaptotagmin, rab3a, and synaptobrevin (A). Cortical proteins were also immunostained with antibodies against syntaxin and SNAP-25 and with the peroxidase-conjugated secondary antibody (B). The secondary antibody used in B differed in that it gave increased background staining. Bound antibodies were visualized using the enhanced chemiluminescence system (ECL, Amersham Int. plc).

Fig. 2. Immunocytochemical staining to show the distribution of the synaptobrevin homologue within the egg cortex. Fixed egg cortices were immunostained using anti-synaptobrevin monoclonal antibody (C1 10.1) and were visualized using a fluorescein-conjugated secondary antibody and confocal microscopy: (A) horizontal section; (B) vertical section. For comparison, unixed cortices were stained with DiIC18: (C) horizontal section; (D) vertical section. Bars: 10 µm (A,C); 2.5 µm (B,D).
A whole egg membrane extract

![Diagram of protein digestion](image)

B isolated egg cortex

![Diagram of protein digestion](image)

---

**Fig. 3.** TeTx LC specifically cleaves the 17.5 kDa synaptobrevin homologue in vitro. (A) A whole egg membrane fraction was incubated either in the presence (upper panel) or absence (lower panel) of 270 nM TeTx LC at 37°C. Samples were taken at the times indicated and proteins extracted, resolved by SDS-PAGE (10 µg protein/lane), electrotransferred to a PVDF membrane and immunostained using monoclonal antibody C1 10.1. (B) Cortices were incubated in either the presence (+) or absence (−) of 270 nM TeTx LC for 15 minutes at 37°C. Cortical proteins were then resolved by SDS-PAGE (20 µg protein/lane) and electrotransferred to a PVDF membrane. The membrane was cut horizontally and each part was immunostained using either anti-synaptobrevin (C1 10.1) or anti-rab3a (C1 42.1) monoclonal antibodies.

---

**Fig. 4.** The effect of temperature on exocytosis in vitro. Ca²⁺-stimulated exocytosis in vitro was measured after incubation at either 16°C or 37°C, for 5 or 15 minutes. After incubation, cortices were stimulated to undergo exocytosis at 16°C by the addition of 5.9 µM Ca²⁺. Cortices were viewed using phase contrast microscopy and exocytosis was measured 1 minute after calcium addition. Mean ± s.e.m. are shown, n=6.

**Fig. 5.** Inhibition of Ca²⁺-stimulated exocytosis in vitro. Egg cortices were preincubated at 37°C for 5 or 15 minutes, either in the presence or absence of 270 nM TeTx LC. Cortices were viewed using phase contrast microscopy. Exocytosis was stimulated by the addition of 5.9 µM Ca²⁺ and was measured 1 minute after Ca²⁺ addition. Mean ± s.e.m. are shown, n=6.

---

protein to be specifically cleaved (Fig. 3A, upper panel). The majority of the protein was digested in the first 15 minutes of incubation and complete digestion was obtained after 30 minutes. In a control incubation, in which no TeTx LC was added, no digestion of the 17.5 kDa protein was observed (Fig. 3A, lower panel). The specific cleavage of the sea urchin 17.5 kDa protein by TeTx LC demonstrates that it is structurally related to mammalian synaptobrevins.

It was previously reported that synaptobrevin is insensitive to TeTx LC cleavage when in the 7 S protein complex proposed to mediate vesicle docking at the plasma membrane (Pellegrini et al., 1994; Hayashi et al., 1994). In order to test this hypothesis, we incubated isolated egg cortices with TeTx LC at 16°C, the physiological temperature of *Lytechinus pictus* eggs. Subsequent protein extraction and western blot analysis revealed that no significant digestion of the 17.5 kDa protein had occurred, even after a two hour incubation (data not shown). However, when the isolated cortex was incubated with TeTx LC at 37°C, complete cleavage of the 17.5 kDa protein was obtained after 15 minutes (Fig. 3B, upper panel). Simultaneous measurement of rab3a immunoreactivity by western blotting indicated that proteolysis by the toxin was specific to the synaptobrevin homologue (Fig. 3B, lower panel).

---

**Treatment of isolated cortices with tetanus toxin inhibits exocytosis in vitro**

As might be predicted from the above experiments, incubation of sea urchin egg cortices with TeTx LC at 16°C did not affect Ca²⁺-induced exocytosis (data not shown). Since the physiological temperature at which experiments on the isolated cortex are usually conducted is 16°C, we first determined whether in vitro exocytosis could take place after incubation of the cortex at 37°C. As shown in Fig. 4, Ca²⁺-induced exocytosis still occurred at 37°C, although the extent of the response was decreased when compared to the 16°C control. Note, though, that some loss of activity is seen over time at both 16°C and...
37°C. This ‘rundown’ effect was previously reported to be due to the dissociation of exocytosis-relevant proteins from the egg cortex (Sasaki, 1992; Moy et al., 1983; Baker and Whitaker, 1978). Therefore, it is likely that the higher temperature merely accelerates processes that are already occurring at 16°C, albeit at a slower rate.

Having established that in vitro exocytosis can occur at elevated temperature, we determined the effects of TeTx LC on in vitro exocytosis at 37°C, the temperature at which we found the toxin to be efficient at degrading the synaptobrevin homologue. We found a significant and time-dependent inhibition of Ca2+-induced cortical granule-plasma membrane fusion (Fig. 5). Incubation with TeTx LC inhibited Ca2+-induced exocytosis by 43% after 5 minutes and by 63% after 15 minutes. We did not see any indication that treatment with toxin caused dissociation of secretory granules from the plasma membrane. These data suggest that the inhibition occurred at a stage after docking but preceding fusion.

**DISCUSSION**

Our experiments indicate that homologues of the mammalian neuronal docking/fusion complex proteins exist in sea urchin eggs and are associated with the egg cortex. We show that the 17.5 kDa synaptobrevin homologue is specifically localized to the cortical granule membrane and is therefore in an analogous location to its neuronal counterpart. Since exocytosis can be stimulated in vitro without cytosolic constituents (Vacquier, 1975; Baker and Whitaker, 1978), these proteins may constitute the necessary and sufficient fusion machinery.

The putative sea urchin homologues of synaptotagmin and synaptobrevin detected by western blotting run at lower molecular masses than their neuronal counterparts in SDS gels (Fig. 1A). Similarly, ubiquitously expressed mammalian isoforms of synaptotagmin and synaptobrevin (synaptotagmin VII, VIII, and cellubrevin, respectively) have smaller molecular masses than the neuron-specific isoforms (Li et al., 1995; McMahon et al., 1993). This suggests a closer structural relationship of the sea urchin proteins to the ubiquitously expressed isoforms, and is consistent with the observation that both the putative sea urchin synaptobrevin homologue and cellubrevin are substrates for TeTx (Fig. 3, McMahon et al., 1993).

Studies of the neuronal fusion proteins in detergent extracts resulted in the identification of a series of sequential protein-protein interactions that were proposed to underly vesicle docking and fusion at the plasma membrane (Sollner et al., 1993). The final step in this sequence, ATP hydrolysis by NSF, causes disassembly of a 20 S putative fusion complex. This disassembly was proposed to be the force driving fusion of the lipid bilayers (Sollner et al., 1993). This model, however, has since been disputed since in most secretory systems, ATP is not required for the final calcium-triggered fusion event. For example, in seaurchin eggs, ATP, although required to prime the exocytotic apparatus, is not required for calcium-triggered fusion per se (Baker and Whitaker, 1978). A modified hypothesis proposed that ATP acts through NSF at a prefusion stage to energize the 7 S complex, preparing it for fast, calcium-triggered membrane fusion (O’Connor et al., 1994). In agreement with this, NSF and α-SNAP were shown to function in a post-docking, ATP-dependent priming step, that preceded the actual calcium-triggered fusion in permeabilized PC12 cells (Banerjee et al., 1996). However, in yeast, NSF and α-SNAP were shown to function in a pre-docking step in in vitro vacuolar fusion (Mayer et al., 1996). Thus, although the identity of proteins that function during exocytosis is well established, the stage at which disassembly of the 20 S complex occurs, and the series of protein-protein interactions that lead to membrane fusion are still unknown. The identification of homologues of the neuronal fusion proteins in sea urchin eggs is of major importance since this system offers the unique opportunity to study, in vitro, both docking and fusion reactions (Crabb and Jackson, 1985). It is possible to separate cortical granules from the plasma membrane. These can then be reassocitated to form exocytotically-competent cortical lawns which undergo fusion upon the addition of micromolar calcium (Crabb and Jackson, 1985). The preparation can thus be used to investigate the effects of treatment of either, or both, of the isolated membrane components with compounds that affect the putative fusion proteins, upon subsequent docking and calcium-stimulated fusion. This will allow unique insights into the molecular mechanisms underlying each of these processes and may help clarify current models of membrane fusion.

**Susceptibility of docked granules to tetanus toxin**

We have shown that the sea urchin egg synaptobrevin homologue is specifically cleaved by TeTx LC at 37°C in both membrane protein extracts and in isolated cortices. However, when the isolated egg cortex was incubated with TeTx LC at 16°C, no cleavage of the synaptobrevin homologue occurred, even after a two hour incubation. At this temperature, TeTx LC had no effect on Ca2+-induced exocytosis in vitro. It was previously reported that tetanus toxin had no effect on exocytosis when microinjected into intact sea urchin eggs held at 21°C (Bi et al., 1995), indicating that the in vitro cortex preparation behaves similarly to the intact egg. However, our results show that incubation of the isolated cortex with TeTx LC at 37°C resulted in a time-dependent, partial inhibition of Ca2+-induced exocytosis. This inhibition correlated with cleavage of the synaptobrevin homologue. Thus, our data show that, at higher temperatures of incubation, docked granules are indeed sensitive to TeTx LC. It was previously shown that artificially undocking granules in intact eggs makes exocytosis susceptible to microinjected tetanus toxin (Bi et al., 1995), implying that undocked granules are alone susceptible to tetanus toxin. This would seem to confirm reports that the proteins of the 7 S complex become resistant to bacterial neurotoxins once the complex is formed (Hayashi et al., 1994; Pellegrini et al., 1994). Our data here show that docked granules become susceptible to tetanus toxin at higher temperatures of incubation. Since the Q10 of enzyme-mediated reactions is around 2 (Schve, 1984), an increase in temperature from 16°C to 37°C would result in a greater than fourfold increase in TeTx LC activity. It would also increase the thermal motion of the fusion proteins which may increase the susceptibility of synaptobrevin to TeTx LC action. It remains to be clarified whether either, or both, of these factors contribute towards this temperature-dependent effect. This question is not trivial; if the effect is only due to an increase in enzymatic activity then it would suggest that syntaxin, synaptobrevin and SNAP-25 are not in the ternary complex proposed to mediate
vesicle docking at the plasma membrane (Söllner et al., 1993). This conclusion would be consistent with recent work on yeast vacuolar fusion and PC12 cells which has shown that NSF-induced disassembly of the 20S fusion complex occurs prior to calcium-triggered fusion (Banerjee et al., 1996; Mayer et al., 1996). It will be possible to isolate the neuronal fusion protein homologues from sea urchin eggs in order to identify possible novel interactions at the post-docking stage. This will provide new insights into the molecular mechanisms underlying membrane fusion reactions.

**Inhibition of calcium-induced exocytosis by tetanus toxin light chain in vitro**

Treatment of the isolated cortex with TeTx LC at 37°C led to proteolysis of synaptobrevin and inhibition of Ca\(^{2+}\)-induced exocytosis. There was no indication that TeTx LC treatment was accompanied by dissociation of cortical granules from the plasma membrane, implying that synaptobrevin is necessary for fusion competence in docked granules but not for tethering the granules to the plasma membrane. They are consistent with the observation that cortical granules apparently redock after artificial undocking and tetanus toxin treatment in intact eggs, while subsequent exocytotic steps are blocked (Bi et al., 1995).

A post-docking role for synaptobrevin has also been proposed in synaptic vesicle exocytosis (Hunt et al., 1994) consistent with the results we present here. Tethering of granules may in fact involve other protein-protein interactions. A direct interaction between synaptotagmin and SNAP-25 was demonstrated which was proposed to mediate vesicle docking in TeTx-treated synapses (Schiavo et al., 1997). This interaction may stabilize docked granules in the TeTx LC-treated sea urchin egg cortices.

It appears from our data that, apparently, complete cleavage of the synaptobrevin homologue can occur after treatment with TeTx LC without complete inhibition of calcium-induced exocytosis. A similar partial inhibition of exocytosis despite apparently complete cleavage of synaptobrevin has also been observed in other secretory systems (Galli et al., 1994; Chilcote et al., 1995). While it is possible that there may be some variability in the susceptibility of individual cortex preparations to toxin inhibition which may account for this finding, it is equally possible that some secretory granules escape the effects of toxin by using other proteins to sustain their fusion competence. In permeabilized MDCK cells, the existence of two independent transport and fusion mechanisms was reported (Ikoen et al., 1995). In these cells, docking and fusion of secretory vesicles with the apical plasma membrane was insensitive to TeTx but required the function of a novel annexin protein, annexin XIIIb. In contrast, the docking and fusion of secretory vesicles with the basolateral plasma membrane was sensitive to TeTx but was independent of annexin function (Ikoen et al., 1995; Fiedler et al., 1995). Interestingly, the occurrence of annexins in sea urchin eggs has been reported (Shen et al., 1994), one of which was localized to the egg cortex and displayed similar properties to annexin XIIIb (J. Avery, unpublished observations). It remains to be determined whether annexins play any role in calcium-stimulated exocytosis in sea urchin eggs.

**Conclusion**

Exocytosis in the sea urchin egg employs similar proteins to those used by mammalian neurons and other secretory cells, demonstrating substantial conservation of exocytotic mechanisms across phylogenetic boundaries. This is potentially a very valuable finding since the sea urchin egg provides a system in which both docking and fusion reactions can be studied in vitro. Clearly, the unique in vitro model provided by the sea urchin egg cortex can be used to elucidate the molecular mechanisms for the control of membrane fusion that will be wholly relevant to mammalian neurons and other secretory cells.

We thank Dr R. Jahn, Dr T. Schäfer and Dr M. Takahashi for the generous gift of antibodies. We also thank Dr T. Schäfer for helpful discussions and Mr M. Aitchison for technical assistance. This work was supported by grants from the Wellcome Trust and the Medical Research Council. A.H. was supported by the Swiss National Science Foundation and the Ciba-Geigy-Jubiläums-Stiftung.

**REFERENCES**


Hunt, J. M., Bommert, K., Charlton, M. P., Kistner, A., Habermann, E.,...
of cDNA for a neuronal cell membrane antigen, HPC-1. J. Biol. Chem. 267, 10613-10619.


(Received 10 January 1997 – Accepted, in revised form, 2 May 1997)