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+}

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It is known that sea-urchin egg cortical-granule exocytosis is inhibited by agents such as *N*-ethylmaleimide (NEM) which modify thiol groups. The fusion-related proteins modified by these agents have yet to be identified, nor is there information regarding the topography of these thiol groups. Furthermore, the step in cortical-granule exocytosis at which these thiol groups participate is unknown. In this study we have investigated the topological properties of, and the temporal requirement for the function of, the fusion-related thiol groups by treating the isolated exocytotic apparatus with high-molecular-mass dextrans and BSA carrying thiol-reactive 3-(2-pyridyldithio)propionate groups. The dextran derivatives inhibited exocytosis. The BSA

derivative was much less inhibitory. Inhibition was reversed by treatment with dithiothreitol. When NEM was added to the dextran-derivative-treated exocytotic apparatus, treatment with dithiothreitol completely reversed inhibition, indicating that the dextran derivatives inhibit by reacting at the NEM-sensitive sites. A pulse of Ca^{2+} applied in the presence of inhibitors did not trigger any fusion following the removal of the inhibitor by dithiothreitol. These data show that the thiol groups, the modification of which by NEM inhibits exocytosis, are exposed to the medium in terms of their accessibility to macromolecules. They also show that the fusion-related thiol groups are required during the Ca^{2+} -dependent stage of exocytosis.

INTRODUCTION

Sea-urchin egg cortical-granule exocytosis is a concerted and massive secretory event triggered at fertilization by a sperm-induced rise in the intracellular free Ca^{2+} concentration [1,2]. It is a particularly good model with which to investigate regulated exocytosis, because the complete fusion machinery can be conveniently isolated and studied [3,4]. Cortical-granule exocytosis requires elevated Ca^{2+} concentrations [5,6], and in this requirement it resembles many other regulated exocytotic events [7–9]. Although studies in intact eggs have suggested the involvement of G-proteins [10,11] and phosphatases [12] in the control of exocytosis, there is no evidence for the involvement of these proteins in exocytosis *in vitro*. These factors are more likely to exhibit their effects through modulation of signal transduction [13] and other events downstream of membrane fusion; in fact, cortical-granule exocytosis *in vitro* seems only to require plasma membrane, cortical granules and elevated levels of Ca^{2+} [14].

Previous studies have described a number of methods to inhibit cortical-granule exocytosis. Among these are the application of inhibitors of calmodulin function to the isolated cortex [5,15], raising the osmotic strength of the bathing solution with low-molecular-mass solutes [16], treating eggs with high-molecular-mass water-soluble polymers [17,18] and pre-treating membranes with low, non-lytic, concentrations of lysolipids [19]. Of these, the last two classes of inhibitors have been particularly well characterized in terms of the position in the stimulus-secretion coupling pathway at which they act. When exocytosis is triggered in the presence of water-soluble polymers, membrane fusion occurs, but dispersion of the granule contents is prevented [17,18]. In contrast, lysolipids arrest exocytosis at a stage before

lipid mixing [20]. This inhibitor appears to prevent exocytosis after activation of the fusion machinery, but before actual membrane merger.

In nerve cells a number of proteins have been suggested to mediate the membrane fusion required during neurotransmission [21]. In contrast, there are no indications as to which proteins might be involved in membrane fusion during cortical-granule exocytosis. It is known, however, that cortical-granule exocytosis [22,23], like other membrane fusion events [24–26], is susceptible to inhibition by *N*-ethylmaleimide (NEM), which implies the involvement of thiol-containing proteins. In the systems in which the targets of inhibition by NEM have been identified, they have been shown to be cytosolic proteins such as NEM-sensitive factor (NSF) in endoplasmic reticulum to Golgi [27] or endocytic-vesicle fusion [28], or ETF-1 in vesicular transport from late endosomes to the *trans*-Golgi network [29]. Cytosol is not necessary for the exocytosis of cortical granules *in vitro* [14,23], nor is either ATP or GTP required [14], which suggests that this exocytotic event appears not to require the enzymic activities of either NSF [30] or G-proteins [31]. It seems likely that the exocytotic fusion machinery may be arrested at a stage which closely precedes the merging of lipid bilayers. If NSF or G-proteins are involved, it is probable that they participate in the earlier ‘docking’ steps which occur during oocyte maturation [32].

The NEM-sensitive proteins involved in cortical-granule exocytosis are present in both the plasma membrane [33] and the cortical granules [23,33]. However, since these proteins are tightly associated with the fusing membranes, the techniques which were so useful in identifying cytosolic NEM-sensitive proteins in other intracellular membrane-fusion events [25] are of little practical

Abbreviations used: NEM, *N*-ethylmaleimide; NSF, NEM-sensitive factor; CSC, cell-surface complex; PDP, 3-(2-pyridyldithio)propionate; SPDP, *N*-succinimidyl PDP; DTT, dithiothreitol.

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use. One of the alternative approaches for the identification of thiol-containing proteins might be the use of inhibitors of fusion with a set of substrates more limited than that of NEM. As a first step in this direction, we have attempted to characterize the topology of the NEM-sensitive proteins by treating sea-urchin egg cell-surface complexes (CSCs: the vitelline membrane, plasma membrane and attached cortical granules) with large dextrans and BSA modified with thiol-reactive 3-(2-pyridyl-dithio)propionate (PDP) groups, which can reversibly react with thiol moieties. We have found that these thiol groups are highly exposed to the medium. We have also used PDP-containing dextrans to define more precisely the role of NEM-sensitive proteins in cortical-granule exocytosis and to determine whether they are important in triggering (initiation of the fusion process), commitment (establishment of a trigger-independent committed pre-fusion state) or dispersion (pore widening and release of the granule contents into the extracellular space). We have determined that the NEM-sensitive proteins are necessary during a Ca^{2+} -dependent stage of exocytosis.

EXPERIMENTAL

Obtaining and handling eggs

Sea urchins of the species *Lytechinus pictus* were purchased from Marinus (Long Beach, CA, U.S.A.) 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 Hepes, 1 mM EDTA, pH 8.0) at a temperature of 14 °C. Eggs were obtained by injecting 0.5 M KCl into the intracoelomic cavity, and the jelly coat was removed by passing through 110 μm nylon mesh. Eggs were maintained at 16 °C until use.

Preparation of CSCs

Eggs were washed three times in artificial sea water and resuspended in PKME at 4 °C (PKME: 50 mM Pipes, 425 mM KCl, 10 mM MgCl_2 , 5 mM EGTA, pH 6.7). The eggs were washed three more times with this medium and were transferred to a Potter homogenizer. CSCs were prepared by homogenization using five or six strokes of a tight-fitting Teflon pestle until microscopic examination showed that no intact eggs remained. After homogenization, CSCs were pelleted at 700 g for 1 min and resuspended in fresh PKME. Centrifugation was repeated until the preparation appeared free of contamination and consisted only of large sheets of plasma membrane and associated cortical secretory granules. CSCs were stored on ice until use.

Preparation of egg S100 cytosol

Eggs were treated as above and were washed twice more with ice-cold PKME containing 2.5 mM ATP, 10 mM dithiothreitol (DTT), 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ pepstatin and 20 $\mu\text{g}/\text{ml}$ leupeptin (cytosol buffer). The eggs were resuspended in an equal volume of cytosol buffer and were gently homogenized by three strokes of a loose-fitting Teflon pestle in a Potter homogenizer. The homogenate was centrifuged at 4 °C for 1 h at 100 000 g. The supernatant (S100) was retained and passed through a 0.22 μm filter and used at a protein concentration of 10 mg/ml.

Synthesis of PDP derivatives

N-Succinimidyl PDP (SPDP) was purchased from Pierce (Rockford, IL, U.S.A.). Amino-dextrans were from Molecular

Probes (Eugene, OR, U.S.A.). We synthesized PDP dextran derivatives with nominal molecular masses of 10, 40 and 70 kDa (D_{10}PDP , D_{40}PDP , D_{70}PDP) and a BSA derivative (BSA-PDP). The molecular masses of the amino-dextrans that we used (information supplied by the manufacturer) were 10 000 Da (average 8800 Da), 40 000 Da (range 35 000–50 000 Da) and 70 000 Da (range 60 000–90 000 Da). Amino-dextrans were dissolved in reaction buffer (0.1 M K_2HPO_4 /0.1 M NaCl, pH 7.5) and were passed through columns of Sephadex G25 in the same buffer. Fractions were collected and the sugar-containing peaks were concentrated by using Centricon 3 or Centricon 30 micro-concentrators (Amicon, Beverly, MA, U.S.A.). The concentration of dextrans was determined by a modification of the method of Monsigny et al. [34], with glucose as a standard. Briefly, to 20 μl portions of dextran-containing samples in microtitre plates was added 20 μl of 6 mg/ml resorcinol and 100 μl of 75% H_2SO_4 . The plates were heated at 90 °C for 30 min, cooled to room temperature, and the A_{450} was read. The concentration was adjusted so that the final amino group concentration was 5 mM. Amino-dextran derivatives were prepared with SPDP in the same buffer containing 10% dimethyl-formamide by adding a 10-fold molar excess of SPDP (relative to the amino groups) with vigorous shaking. After 30 min the solutions were passed through Sephadex G25 and the sugar-containing fractions were concentrated as above. A PDP derivative of BSA was prepared in essentially the same manner. The final content of PDP in the derivatives was determined by taking 20 μl portions of the solutions and adding 550 μl of reaction buffer. To this was added 50 μl of 100 mM Tris, pH 7.7, containing 50 mM DTT. DTT reacts with PDP to release thiopyridine in stoichiometric quantities. The concentration of thiopyridine was calculated by measuring A_{343} by using a molar absorption coefficient of 8.08×10^3 [35]. The final ratio of glucose monomers to PDP groups was determined to be 14.9 for D_{10}PDP , 36.3 for D_{40}PDP and 33.6 for D_{70}PDP .

Treatment of CSCs

CSCs were suspended in 1 ml of PKME to give an A_{405} of 0.4–0.6. PDP derivatives or NEM (0.5 M in methanol) were added to give the desired final concentrations. Controls contained the same concentration of methanol. Incubations were carried out at 16 °C. After incubation, 5 ml of PKME was added and the CSCs were pelleted by centrifugation at 700 g. This was repeated to ensure removal of all traces of inhibitor that had not reacted. When reversibility was tested, DTT was added from a 1 M stock to give a final concentration of 50 mM. CSCs were resuspended in PKME, and 100 μl portions were dispensed into flat-bottomed microtitre plates (Costar, Cambridge, MA, U.S.A.). Exocytosis was triggered by addition of 100 μl of Ca^{2+} /PKME to give the required Ca^{2+} concentrations [23]. Ca^{2+} concentrations were verified by using a Ca^{2+} -selective electrode (World Precision Instruments, Sarasota, FL, U.S.A.). Exocytosis was determined by measuring A_{405} with a ThermoMax microtitre-plate reader (Molecular Devices, Menlo Park, CA, U.S.A.) as previously described [23]. The extent of exocytosis was normalized by using A_{405} in PKME containing 0.1 μM free Ca^{2+} as 0% exocytosis and A_{405} in PKME containing 1 mM free Ca^{2+} as 100% exocytosis. Higher Ca^{2+} concentrations caused no further change in A_{405} (results not shown). We determined that a linear relationship existed between the percentage of reaction and percentage change in A_{405} by mixing ratios of CSCs that had not reacted with fully reacted CSCs (quenched with EGTA) and measuring A_{405} (results not shown). In each experiment, all determinations were made in duplicate.

Labelling CSCs with [^3H]NEM

[^3H]NEM (55 Ci/mmol) was purchased as a solution in pentane (New England Nuclear, Boston, MA, U.S.A.). It was taken into aqueous solution by mixing equal volumes of deionized water and [^3H]NEM-containing pentane. Immediately before use, the mixture was vigorously agitated for 30 s by vortex-mixing, and the upper phase was evaporated by the passage of a gentle stream of N_2 . CSCs were suspended in 0.5 ml of PKME together with the desired concentration of PDP derivatives. After the required time, the volume was increased to 5 ml and the CSCs were pelleted. Washing was repeated three times and the CSCs were resuspended to 1 ml in PKME. Then 10 μCi of [^3H]NEM was added to each tube and the CSCs were incubated for 1 h. After this time the CSCs were washed four times in PKME. Proteins were then precipitated by addition of an equal volume of 25% (w/v) trichloroacetic acid. The precipitate was washed twice in acetone/water (4:1, v/v) and the pellet was dissolved in 100 μl of 1 M NaOH. [^3H]NEM incorporation was determined by liquid-scintillation counting.

RESULTS

Thiol-modifying agents inhibit exocytosis in *Lytechinus pictus* egg CSCs

We investigated the effects of thiol-modifying agents on Ca^{2+} -dependent exocytosis in CSCs derived from eggs of the sea urchin *Lytechinus pictus*. The first compound that we tested was NEM. CSCs were incubated with different concentrations of NEM for 2 h, after which they were assayed for exocytosis in response to various Ca^{2+} concentrations. Exocytosis in control CSCs (incubated for 2 h) is typically initiated at 3 μM Ca^{2+} and is half-maximal at about 25 μM , values similar to those published previously for exocytosis in KCl-containing media [36]. NEM concentrations above 300 μM inhibit exocytosis by increasing the Ca^{2+} concentration required for fusion. Higher concentrations of NEM also decrease the extent of exocytosis (Figure 1a). We wanted to test whether the inhibition by NEM could be overcome by the addition of cytosolic proteins, and thus if there was a degree of functional homology between cortical-granule exocytosis and membrane fusion events which require NEM-sensitive cytosolic proteins [25]. Incubations for 30 min with high concentrations (10 mg/ml) of cytosol were unable to restore any exocytotic activity to CSCs previously inactivated by treatment with 5 mM NEM, nor did treatment with cytosol have any effect on exocytosis in control CSCs. The provision of ATP (2.5 mM), GTP (100 μM) and DTT (50 mM) together with cytosol was similarly without effect (results not shown).

In an attempt to obtain a more specific inhibitor of exocytosis, we studied whether exocytosis was inhibited by the bulky hydrophilic thiol-specific reagent D_{10}PDP . CSCs were treated with a range of concentrations of D_{10}PDP for 2 h and Ca^{2+} -dependent exocytosis was measured. D_{10}PDP inhibited exocytosis in essentially the same manner as NEM, that is by increasing the Ca^{2+} required for triggering (Figure 1b). Inhibition by D_{10}PDP (which reacts with thiols to produce disulphide bonds), but not by NEM (an alkylating agent), was reversible by treatment with the reducing agent DTT (Figure 1c). Treatment with 50 mM DTT for 10 min had no effect on inhibition by NEM, but completely reversed the inhibition by D_{10}PDP , indicating that its inhibitory effect is due only to reversible thiol-group modification.

We tested whether other PDP derivatives could inhibit Ca^{2+} -stimulated exocytosis. CSCs were incubated with the PDP-dextrans at 200 μM for 2 h and exocytosis was measured. Each

of the PDP-dextrans was capable of inhibiting exocytosis, with D_{10}PDP the most potent inhibitor. D_{40}PDP and D_{70}PDP were similar in their effects on exocytosis (Figure 2). Like the inhibition by NEM, inhibition by PDP-dextrans increased the Ca^{2+} concentration required for exocytosis and, at high concentrations also decreased the extent of exocytosis. Compared with the PDP-dextrans, BSA-PDP was less inhibitory. At the pH of our experiments, BSA-PDP is negatively charged, in contrast with the PDP-dextrans, which are either neutral or slightly positively charged. This may affect its ability to interact with certain

Figure 1 Thiol-modifying reagents inhibit cortical-granule exocytosis in *Lytechinus pictus* egg CSCs

(a) CSCs were treated with NEM for 30 min. A Ca^{2+} -dose/response curve was constructed and exocytosis determined by measuring A_{405} as described in the Experimental section. Symbols: $\circ$, control; $\bullet$, 312 μM ; ∇ , 625 μM ; $\blacktriangledown$, 1.25 mM; $\square$, 2.5 mM; $\blacksquare$, 5 mM NEM. Each point represents the mean $\pm$ S.E.M. ($n = 3$). (b) CSCs were treated with D_{10}PDP for 2 h and Ca^{2+} -stimulated exocytosis was determined as above. Symbols: $\circ$, control; $\bullet$, 12.5 μM ; ∇ , 25 μM ; $\blacktriangledown$, 50 μM ; $\square$, 100 μM ; $\blacksquare$, 200 μM D_{10}PDP . Means $\pm$ S.E.M. are shown ($n = 3$). (c) CSCs were treated with 5 mM NEM for 30 min or with 200 μM D_{10}PDP for 2 h. CSCs were washed and resuspended in PKME, and the extent of exocytosis was determined before and after treatment with 50 mM DTT for 10 min. Symbols: $\circ$, 200 μM D_{10}PDP ; $\bullet$, 200 μM D_{10}PDP + 50 mM DTT; ∇ , 5 mM NEM; $\blacktriangledown$, 5 mM NEM + 50 mM DTT. Means $\pm$ S.E.M. are shown ($n = 3$).

Figure 2 Inhibition of Ca^{2+} -stimulated cortical-granule exocytosis by PDP derivatives is related to the size of the derivative

We determined whether a relationship existed between the size of each PDP derivative and its ability to inhibit Ca^{2+} -stimulated exocytosis. CSCs were incubated with each derivative at a concentration of $200 \mu\text{M}$ (with respect to the thiol-reactive moiety). After 2 h, CSCs were resuspended in fresh PKME and exocytosis was determined. Symbols: ●, control; ○, D_{10}PDP ; ◐, D_{70}PDP ; ▼, BSA-PDP. Means $\pm$ S.E.M. are shown ($n = 3$).

Table 1 Derivatives of PDP decrease $[^3\text{H}]\text{NEM}$ incorporation into CSCs

CSCs ($A_{405} 0.6$) were suspended in PKME and were incubated for 2 h with PDP derivatives ($200 \mu\text{M}$) or just PKME (control). After 2 h the CSCs were washed three times by centrifugation and resuspension, and were then incubated for 1 h with $[^3\text{H}]\text{NEM}$ ($10 \mu\text{Ci}/\text{tube}$). CSCs were washed four times in PKME, and protein was precipitated by addition of an equal volume of 25% trichloroacetic acid. The pellet was washed twice in acetone/water (4:1, v/v) and the pellet dissolved in $100 \mu\text{l}$ of 1 M NaOH. $[^3\text{H}]\text{NEM}$ incorporation was determined by liquid-scintillation counting. Means $\pm$ S.E.M. are shown ($n = 3$).

Condition	$10^{-3} \times [^3\text{H}]\text{NEM}$ incorporation (d.p.m./mg of protein)
Control	1850 ± 370
D_{10}PDP	512 ± 47
D_{70}PDP	611 ± 33
D_{70}PDP	762 ± 10
BSA-PDP	665 ± 113

membrane domains. To determine if BSA-PDP was capable of interacting with at least some membrane thiol groups, we measured how much each PDP derivative could decrease the incorporation of $[^3\text{H}]\text{NEM}$ into CSC proteins. CSCs were incubated for 2 h with inhibitors; they were then washed, resuspended and labelled with $[^3\text{H}]\text{NEM}$ ($10 \mu\text{Ci}/\text{ml}$) and radioactivity incorporation was determined by liquid-scintillation counting. We found that the incorporation of $[^3\text{H}]\text{NEM}$ into trichloroacetic acid-precipitable CSC protein was decreased by approx. 75% in CSCs pretreated with each inhibitor (Table 1). This large decrease in incorporation of the label reflects the participation in the reaction of only the most exposed CSC thiol groups. This rules out the possibility that BSA-PDP could not interact with membrane surfaces in a manner required for the reaction between the PDP residues of modified BSA and the thiol groups of CSC proteins.

PDP derivatives inhibit exocytosis at the same sites as NEM

Both NEM and the PDP derivatives inhibit exocytosis by increasing the Ca^{2+} concentration required for the onset of

Figure 3 D_{10}PDP protects CSCs from inhibition by NEM

We tested whether D_{10}PDP and NEM inhibited exocytosis through the same site by determining whether D_{10}PDP could protect Ca^{2+} -stimulated exocytosis against inhibition by NEM. Symbols: ○, control CSCs; ▼, CSCs were treated with $200 \mu\text{M}$ D_{10}PDP for 2 h, followed by 5 mM NEM for 30 min; ●, CSCs were treated with $200 \mu\text{M}$ D_{10}PDP for 2 h. They were washed free of D_{10}PDP , resuspended in PKME and treated with 5 mM NEM for 30 min. After this, CSCs were washed once more and treated with 50 mM DTT for 10 min. Ca^{2+} -stimulated exocytosis was determined. Means $\pm$ S.E.M. are shown ($n = 4$).

fusion, which suggests that the modification of a common target might be behind this inhibition. It is also possible that PDP derivatives might react with only a subset of the sites which are alkylated by NEM, which, because it is more hydrophobic and of lower molecular mass, might be expected to have access to additional reactive sites. We investigated this possibility by testing whether treatment of CSCs with inhibitory concentrations of PDP derivatives protected them from subsequent inhibition by NEM. If the fusion-related sites at which NEM reacts are blocked by PDP derivatives, CSCs treated with PDP derivatives and then by NEM should still be competent to fuse in response to Ca^{2+} following treatment with DTT. We found that CSCs treated with 50 mM DTT for 10 min after incubation with $200 \mu\text{M}$ D_{10}PDP followed by 5 mM NEM had an exocytotic response very similar to that of untreated CSCs (Figure 3). This result shows that D_{10}PDP protects the fusion-related thiol groups from alkylation by NEM and that under these conditions the mechanism of inhibition by both of these inhibitors is by modification of identical reactive sites. In confirmation of this finding is the observation that the degree of protection afforded by the other PDP derivatives is directly related to their inhibitory potency (results not shown).

D_{10}PDP inhibits exocytosis before commitment

It has been shown previously that both water-soluble polymers [17] and lysolipids [20] inhibit exocytosis at a stage after triggering by Ca^{2+} . We used D_{10}PDP to define the point in the pathway between Ca^{2+} -triggering and granule-content dispersion at which NEM-sensitive proteins play a necessary role. CSCs were inhibited with D_{10}PDP and were exposed to triggering Ca^{2+} concentrations. After 2 min the Ca^{2+} concentration was returned to non-triggering levels by the addition of EGTA. When D_{10}PDP was removed by treatment with DTT, no fusion resulted (Figure 4). Thus there was no 'memory' of the Ca^{2+} trigger. We showed that the inhibition by D_{10}PDP could still be reversed by adding 50 mM DTT 2 min after triggering by Ca^{2+} without first decreasing Ca^{2+} to non-stimulatory levels. Exocytosis under these circumstances was similar to that in control CSCs. We also found that reversal of inhibition was possible after giving a pulse

Figure 4 D_{10}PDP -treated CSCs are not committed to fusion

We used D_{10}PDP to define the role of NEM-sensitive proteins in cortical-granule exocytosis. We tested whether CSCs were committed to fuse after Ca^{2+} treatment in the presence of D_{10}PDP . ●, Control CSCs treated with only 50 mM DTT for 10 min before determining Ca^{2+} -stimulated exocytosis. ▼, CSCs were treated with 200 μM D_{10}PDP for 2 h. After this, they were exposed to a series of Ca^{2+} concentrations. After 2 min, sufficient 200 mM EGTA (in PKME) was added to lower the Ca^{2+} concentration to less than 200 nM. DTT (from a 1 M stock in 50 mM Pipes/425 mM KCl/10 mM MgCl_2 , pH 6.7) was added to give a final concentration of 50 mM, and the extent of exocytosis was assessed after a further 10 min. ▽, To test for the reversibility of inhibition by D_{10}PDP after exposure to Ca^{2+} , CSCs were treated with 200 μM D_{10}PDP for 2 h, followed by the addition of Ca^{2+} . After 2 min, 50 mM DTT was added and exocytosis measured 10 min later. Means $\pm$ S.E.M. are shown ($n = 4$).

of Ca^{2+} , reducing with DTT and giving a second pulse of Ca^{2+} (results not shown). These data suggest that the role of the NEM-sensitive protein is at or before the triggering of exocytosis by Ca^{2+} , and that the inhibition of fusion caused by agents which inactivate this protein occurs before membrane merger.

DISCUSSION

Little is known of the proteins involved in mediating the complex topological transition from two bilayers to a single continuous bilayer which must occur during cortical-granule exocytosis. One of the few clues regarding these proteins is that they are susceptible to inhibition by reagents such as NEM which modify protein thiol groups [22,23,37,38]. We report here that the exocytosis of *Lytechinus pictus* egg cortical granules is also inhibited by bulky membrane-impermeant derivatives of PDP, an agent which can make reversible disulphide bonds [35]. In contrast with the inhibition caused by NEM, inhibition by D_{10}PDP is completely reversed by treatment with DTT. This is consistent with the only effect of PDP inhibitors being the formation of new disulphide bonds. The choice of dextrans as carriers for PDP groups is based on a few of their characteristics. Firstly, the biologically uncommon α -1,6 bonds between the glucose residues of dextrans are resistant to most cellular glycosidases. Secondly, dextrans appear not to bind to intracellular components [39]. These first two traits make dextrans ideal non-interacting carriers of the thiol-reactive PDP residues. The third important characteristic is that dextrans with molecular masses greater than 2 kDa behave as almost perfect statistical coils in aqueous solutions [40], with a persistence length predicted to be only 0.6–1.3 nm [41]. Rod-like conformations appear to exist only for dextrans with molecular masses less than 2 kDa [40]. Therefore, in terms of their interactions with other macromolecules, high-molecular-mass dextrans can be described as

globular molecules with limited flexibility [42]. This imposes certain exposure requirements on the sites to which the dextrans can have access. That only certain thiol groups in membrane-associated proteins are accessible to PDP-dextrans has been shown in control experiments using rat liver microsomes, with which haemoglobin associates during preparation. The *b*-chain of haemoglobin, which is highly reactive towards NEM, due to one relatively exposed thiol group [43], failed to react to any detectable extent with D_{10}PDP (results not shown).

We have tried to gain more information regarding the topography of the important fusion-related thiol groups by studying the inhibition of exocytosis by thiol-reactive molecules of different sizes. We found that fusion was inhibited by PDP derivatives as large as 60 kDa (the lowest molecular-mass value for D_{70}PDP is 60 000; see the Experimental section). Although we cannot rule out the possibility that inhibition by PDP-derivatives is due in part to a non-specific coating of the two interacting membrane surfaces (plasma membrane and secretory granule), it seems unlikely, given that the PDP-dextrans protected the fusion activity against inhibition by NEM. D_{10}PDP completely obviated the inhibitory effects on exocytosis of NEM. For D_{40}PDP and D_{70}PDP the protective effects were in direct proportion to their inhibitory effects. This shows that all of the fusion-related thiols which are alkylated by NEM are also reactive towards PDP groups, and strongly suggests that the PDP derivatives inhibit exocytosis by reacting with these thiol groups. Thus the NEM-sensitive fusion-related proteins can be defined as a subset of the thiol-containing CSC proteins which can react with PDP-dextrans.

There are a number of factors which we might consider in discussing the differences in the inhibitory potencies of the PDP-dextrans. The first factor is the size of the dextran molecules; the second, the density of PDP groups on the surface of the dextran molecules; and the third, the concentration of dextrans in the incubation mixture. Since these parameters differ for each of the PDP-dextrans used in this study, the exact correlation between the inhibitory effects and the molecular characteristics of the PDP-dextrans is difficult to determine. Qualitatively, however, our results indicate that the modified fusion-related thiol groups are in an exposed hydrophilic environment. Given that the dextrans that we used behave in solution as random coils, the differences in inhibitory effects between D_{10}PDP and the other PDP-dextrans might be attributed to the different radii of the PDP-dextran molecules. The reason for the difference in inhibition between D_{70}PDP and BSA-PDP is unclear.

Previous studies have identified inhibitors whose modes of action have been thoroughly examined. The first class of compounds are water-soluble polymers which inhibit exocytosis by preventing the dispersion of the granule contents [17,18], and thus inhibition occurs at a stage later than membrane merger. This inhibition appears to be due to the prevention of water movement into the granule matrix, which is a normal requirement for content discharge [18]. Lysolipids inhibit fusion after activation of the fusion machinery, but before the establishment of bilayer continuity [20]. We have used D_{10}PDP to define the position in the stimulus-secretion pathway at which NEM-sensitive proteins have a necessary function. Inhibition by either NEM or D_{10}PDP is characterized initially by an increase in the threshold Ca^{2+} concentration required to initiate fusion without affecting the extent of fusion. At higher inhibitor concentrations, however, there is also a decrease in the extent of the secretion in response to 1 mM Ca^{2+} . These results are in keeping with the suggestion that NEM-sensitive proteins are involved in exocytosis at the Ca^{2+} -dependent stage [22,37]. When the exocytotic apparatus is exposed to a pulse of Ca^{2+} in the presence of D_{10}PDP ,

there is no 'memory' of the trigger after reversing the effect of the inhibitor. This is in contrast with the inhibition by lysolipids, where membrane fusion would have occurred under similar experimental conditions because of the establishment of a trigger-independent committed pre-fusion state [20]. Polymers arrest exocytosis at a stage after fusion but before content dispersion [17,18], and again, the removal of the inhibitor after triggering would be expected to lead to complete exocytosis. This result further supports the idea that the NEM-sensitive proteins are required during or before the triggering step. We now have reversible inhibitors for each of three steps of the exocytotic pathway in sea-urchin egg cortical-granule exocytosis. Triggering is inhibited by NEM or PDP derivatives, membrane merger (but not the activation of the fusion machinery) is inhibited by lysolipids, and content dispersion can be inhibited by applying the exocytotic trigger in the presence of water-soluble polymers.

Based on our current level of understanding, it seems unlikely that the NEM-sensitivity of cortical-granule exocytosis and that observed in the vesicular trafficking pathways share a similar structural or functional basis. Sea-urchin egg cortical-granule exocytosis does not involve a recruitment of cytosolic proteins [14,23] or ATP hydrolysis [44,45], unlike the scheme described for NSF-driven fusion [46], and we have shown here that exocytosis cannot be restored by treatment of NEM-inhibited CSCs with high concentrations of egg cytosol. Furthermore, our observation that the NEM-sensitive step occurs before or during the triggering step contrasts with the hypothesis [46] that, in regulated exocytosis, the triggered release of an inhibitory protein such as synaptotagmin [47] occurs before NSF binding and ATP-dependent membrane fusion [46]. Other plausible interpretations for the role of NSF in triggered exocytosis have been proposed [48]. The finding that the role of NEM-sensitive proteins in the control of cortical-granule exocytosis is in the triggering of the fusion process in response to elevated Ca^{2+} concentrations leads us to speculate that the protein might be the Ca^{2+} -receptor or the fusion protein itself. Using our knowledge of their location [23,33,34], together with a judicious application of thiol-reactive compounds, the identification, purification and characterization of the crucial NEM-sensitive proteins required for cortical-granule exocytosis should be possible and will allow us to understand the mechanism of exocytosis more completely.

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