

Exocytosis Induction in *Paramecium tetraurelia* Cells by Exogenous Phosphoprotein Phosphatase In Vivo and In Vitro: Possible Involvement of Calcineurin in Exocytotic Membrane Fusion

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Abstract. Since it had been previously shown that in *Paramecium* cells exocytosis involves the dephosphorylation of a 65-kD phosphoprotein (PP), we tried to induce exocytotic membrane fusion by exogenous phosphatases (alkaline phosphatase or calcineurin [CaN]). The occurrence of calmodulin (CaM) at preformed exocytosis sites (Momayezi, M., H. Kersken, U. Gras, J. Vilmart-Seuwen, and H. Plattner, 1986, *J. Histochem. Cytochem.*, 34:1621-1638) and the current finding of the presence of the 65-kD PP and of a CaN-like protein in cell surface fragments ("cortices") isolated from *Paramecium* cells led us to also test the effect of antibodies (Ab) against CaM or CaN on exocytosis performance.

Microinjected anti-CaN Ab strongly inhibit exocytosis. (Negative results with microinjected anti-CaM Ab can easily be explained by the abundance of CaM.) Alternatively, microinjection of a Ca²⁺-CaM-CaN complex triggers exocytosis. The same occurs with alkaline phosphatase. All these effects can also be mimicked in

vitro with isolated cortices. In vitro exocytosis triggered by adding Ca²⁺-CaM-CaN or alkaline phosphatase is paralleled by dephosphorylation of the 65-kD PP. Exocytosis can also be inhibited in cortices by anti-CaM Ab or anti-CaN Ab.

In wild-type cells, compounds that inhibit phosphatase activity, but none that inhibit kinases or proteases, are able to inhibit exocytosis. Exocytosis cannot be induced by phosphatase injection in a membrane-fusion-deficient mutant strain (*nd9-28°C*) characterized by a defective organization of exocytosis sites (Beisson, J., M. Lefort-Tran, M. Pouphe, M. Rossignol, and B. Satir, 1976, *J. Cell Biol.*, 69:126-143). We conclude that exocytotic membrane fusion requires an adequate assembly of molecular components to allow for the dephosphorylation of a 65-kD PP and that this step is crucial for the induction of exocytotic membrane fusion in *Paramecium* cells. In vivo this probably involves a Ca²⁺-CaM-stimulated CaN-like PP phosphatase.

PROTEIN phosphorylation is one mechanism thought to be involved in the regulation of membrane fusion during exocytosis (12, 13, 45, 54, 56, 59, 63). Partial proteolysis is another one (27, 36, 44). Furthermore, some fusion-relevant proteins may bind to secretory organelles (1, 7, 8). Yet so far no particular fusion regulation protein has been identified in eukaryotic cells.

Most work with secretory cells shows that a variety of cellular proteins become phosphorylated in response to a trigger agent (6-8, 12, 54, 56, 63). Some of them are represented by receptors (24) or ion channels (14) in the cell membrane, others are located inside the cell (ribosomal [15], microtubular [58], or other cytoskeletal components [45]), and, thus, cannot account for fusion regulation. A third group is cytosolic and becomes attached to secretory vesicles in response to an increased intracellular free calcium concentration (7, 8).

Occasionally, the dephosphorylation of certain phosphoproteins (PP)¹ has also been observed in response to exocytosis triggering (2, 10, 11, 59). This holds particularly true for our system, the *Paramecium* cell, a ciliated protozoon, in which only one 65-kD large PP is dephosphorylated in response to different secretory stimuli (18, 69). Dephosphorylation is very rapid (≤ 1 s, i.e., within the time of actual exocytosis performance) and is rapidly (10-30 s) reversed (69). Therefore, it is difficult to decide under conditions of non-synchronous exocytosis (as in most other systems) whether phosphorylation or dephosphorylation would be the primary phenomenon. Nevertheless, the dephosphorylation of the 65-kD PP has also not yet been unequivocally proved to "ignite" exocytotic membrane fusion in *Paramecium* cells.

1. *Abbreviations used in this paper:* Ab, antibodies; AED, aminoethyl-dextran; CaM, calmodulin; CaN, calcineurin; CaN A and CaN B, calcineurin subunits A and B; PP, phosphoprotein.

Table I. Effects of Different Antibodies, Phosphatase Inhibitors, and Protease Inhibitors on Exocytosis Performance In Vivo and In Vitro*

Experiments	ID ₅₀	ID ₁₀₀
In vitro		
Anti-CaN Ab	1:80 (dilution) = 0.10 mg/ml	—
Anti-CaM Ab	1:350 (dilution) = 0.035 mg/ml	—
Preimmune serum	No effect	—
Protease inhibitor cocktail	No effect	—
In vivo		
Heparin	53 µg/ml ± 7%	160 µg/ml ± 0
Poly-L-glutamate	15 µg/ml ± 4%	35 µg/ml ± 0
Poly-L-aspartate	4 µg/ml ± 5%	16 µg/ml ± 0
Protease inhibitor cocktail (microinjected)		
AED (exogenous)	No effect	—

* For in vitro tests the trigger procedure and its evaluation was as indicated in Fig. 5. In vivo tests were made with exogenous AED added (see Materials and Methods). ID₅₀ and ID₁₀₀ values were derived from the curves obtained with graded concentrations of inhibitors or Ab.

Our present approach seems to promise some more conclusive evidence along these lines. We have tried to induce exocytosis by the application of exogenous phosphatases, either by microinjection or by the use of cell surface complexes ("cortices") that we had recently established as an in vitro model system (65). Our previous demonstration of calmodulin (CaM) binding to the preformed exocytosis sites in *Paramecium* (43) has led us to use calcineurin (CaN) as a Ca²⁺-CaM-dependent PP phosphatase with broad substrate specificity (30, 38, 47–49). We have also shown the presence of a CaN-like protein in our system. Another important tool has been the application of antibodies (Ab) to intact cells (microinjection) or to cortex preparations. Since the 65-kD PP can be shown to be dephosphorylated under these conditions, we conclude that a Ca²⁺-CaM-CaN-mediated 65-kD PP dephosphorylation step might initiate membrane fusion during exocytosis. A CaN-like protein was also identified in the exocrine pancreas on the basis of functional aspects, although its molecular mass is quite different from that of CaN (9). CaN-like proteins have also recently been detected in sea urchin eggs (26). The data presented here are the first conclusive demonstration of the relevance of CaN or CaN-like proteins for exocytosis regulation.

Materials and Methods

Cell Materials

Paramecium tetraurelia cells, strains *K401*, *7S* (wild type), and *nd9-28°C*, a mutation isolated by J. Beisson (Centre National de Recherche Scientifique, Gif-sur-Yvette, France) that is unable to secrete its numerous trichocysts attached to the cell membrane (3) were cultivated as before (52). Aminoethyl-dextran (AED) was prepared and used to induce massive trichocyst exocytosis as in references 50 and 52.

Microinjections

Cells were microinjected as in reference 29. The injection volume was estimated to be ~10% of the cell volume. Injected compounds, dissolved in 10 mM Tris-HCl buffer, pH 7.4 (29), were: alkaline phosphatase (type 567744 with a pH optimum at 7.6; Boehringer, Mannheim, Federal Republic of Germany; cf. reference 62), CaM isolated from *P. tetraurelia* (provided by Dr. J. Schultz, Tübingen; cf. references 31 and 66), CaN from bovine brain (30, 38), CaCl₂, anti-CaM Ab produced in rabbits against performic acid-treated CaM from *P. tetraurelia* (43), and anti-CaN Ab. All Ab were affinity-purified IgG.

The amount of microinjected proteins was generally 100 µg/ml (final concentration in the cell), except for CaM (80 µg/ml). Ca²⁺ was injected to give a calculated final concentration of 10⁻⁵ M. Some injections were also made with a cocktail of protease inhibitors added (6 µg/ml phenylmethylsulfonyl fluoride, freshly dissolved; 13 µg/ml *p*-tosyl-L-arginin-methyl-ester; 5 µg/ml leupeptin; and 0.313 mg/ml aprotinin, all from Sigma Chemical Co., St. Louis, MO; cf. reference 21). After antibody-injection exocytosis was triggered by adding 0.005% wt/vol AED to the outside medium. In microinjection experiments, all cells reacted quite uniformly; the number of trichocysts released was counted under the microscope. The number of injected cells was at least 10 per experiment and three sets of experiments were made in each case. For controls we also injected injection buffer, 10⁻⁵ M Ca²⁺ or CaM alone. In no case could we achieve the release of more than 10–20 trichocysts under any of these control conditions.

Inhibitor Experiments with Cells

Cells were also exposed to inhibitors of phosphatase activity, like heparin (porcine intestinal mucosa, 176 USP/mg; Sigma Chemical Co., see reference 17). We also tested polyglutamate (GLU_{n=70}) and polyaspartate (ASP_{n=150}), both from Sigma, and we found them to be potent inhibitors of AED-induced exocytosis (see Table I). In addition we explored any possible effects of the protein kinase inhibitors diazepam (13) from Serva (Heidelberg, Federal Republic of Germany) and alloxan (directed against Ca²⁺-CaM-dependent protein kinase [46]), as well as of the metalloprotease inhibitor phenanthroline (44) from Janssen Pharmaceutica (Beerse, Belgium). They were applied in concentrations up to 10 times above those indicated in the literature, combined with subsequent AED triggering.

Isolation of Cell Cortex Preparations

Cortices, consisting of the cell surface membrane complex (cell membrane with alveolar sacs) with trichocysts still attached were prepared essentially as previously described (29, 43, 65). We could also unequivocally ascertain by electron microscopy that the triggering of exocytosis in vitro involves genuine membrane fusion and that membrane retrieval is not involved (65). In the present study cortices were produced in two modifications. For testing inhibitors of exocytosis they were preferentially kept in 0.5 mM MgCl₂ (formula a), whereas for testing stimulative agents, 5 mM MgCl₂ was preferentially used (formula b). Both were used in the presence of 10⁻⁵ M free Ca²⁺ (determined with a radiometer type 2112 Ca²⁺-selective electrode), dissolved in 5 mM Tris-maleate buffer, pH 7.0. Formula a or b allowed for a maximal response either under inhibitory or under stimulative conditions, respectively, and these data were normalized as 100% reference values to which the effect of stimulative or inhibitory agents were referred. 30 cortices were evaluated in three independent sets of experiments. Trichocysts released were counted in a phase-contrast microscope. Some experiments with cortices were run with exogenous CaM and/or CaN added, and occasionally with a cocktail of protease inhibitors (see above). For controls we applied preimmune sera or unspecific IgG (against ovalbumin). Pilot experiments were also performed with various proteases, and with phospholipase C (see Discussion).

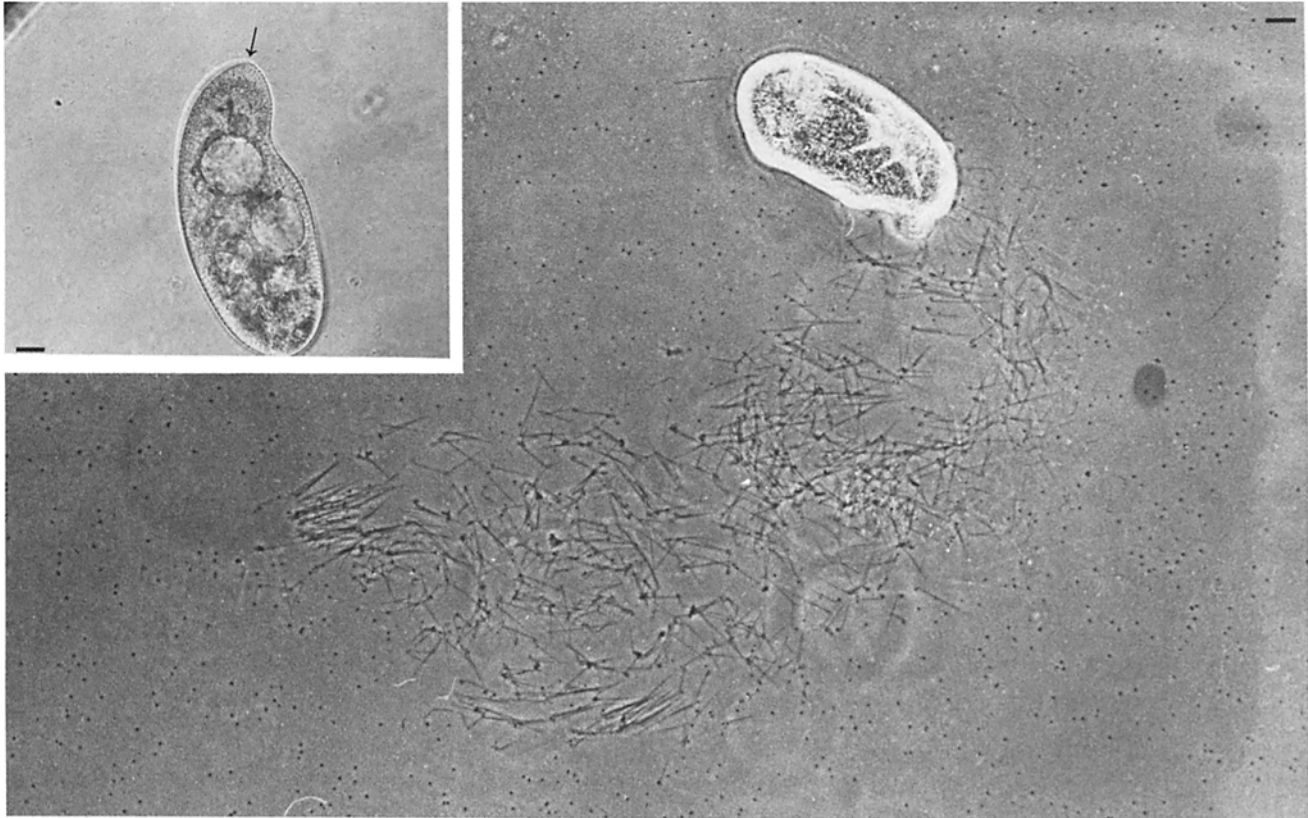


Figure 1. Wild-type cell after injection of alkaline phosphatase (0.1 mg/ml). Exocytosis (with expelled trichocysts visible as ~15- μ m-long rods outside the cell) starts ~30 s after injection and ends ~2 min after injection, when this micrograph was taken. (*Inset*) Exocytosis-deficient *nd9-28°C* cell 5 min after microinjection of alkaline phosphatase. No exocytosis occurs; trichocysts remain visible within the cell cortex as fine rods (e.g., at the *arrow*). Bars, 10 μ m.

Protein Dephosphorylation In Vitro

Cortices were prepared from 32 P-prelabeled cells (69) and exposed to alkaline phosphatase (50 μ g/ml) or to a Ca^{2+} -CaM-CaN (27 or 130 μ g/ml, respectively) complex to trigger exocytosis in vitro (see above). The occurrence of a dephosphorylation of the 65-kD PP was analyzed with methods that have been described before (69). For controls, cortices were kept in the isolation medium, in Ca^{2+} (10^{-5} M), or in CaM (27 μ g/ml) + Ca^{2+} .

RIA

The competitive RIA was performed according to reference 22. CaM or CaN were bound to polystyrene tubes; increasing amounts of cell homogenates were added, followed by the respective Ab. After washing, 125 I-protein A (New England Nuclear, Braunschweig, Federal Republic of Germany) was added.

Identification of CaN-like Protein(s)

Paramecium, whole cells, or isolated cortices were suspended in 50 mM Tris-HCl buffer, pH 8, containing 6 M urea, 0.5 M dithiothreitol, and 1% SDS, to a final concentration of 1.6 mg protein/ml. The suspension was boiled for 3 min and aliquots of solubilized proteins subjected to SDS-PAGE in 5–10% gradients of acrylamide. Polypeptides were then transferred to 0.1- μ m nitrocellulose filters. CaN-like protein(s) was identified by three different methods. (a) Western blots were performed according to the method of Towbin et al. (64) using rabbit anti-(bovine brain) CaN IgG or control rabbit IgG (20 μ g/ml) and 125 I-protein A as a detector for the immune complexes. (b) 125 I-CaM binding to CaN subunit A (CaN A) was measured by the overlay method (30) adapted to proteins transferred to nitrocellulose filters (Hubbard, M., and C. B. Klee, manuscript submitted for publication). After transfer, the filters were quenched with 1% wt/vol β -lactoglobulin in buffer C for 30 min at 40°C, incubated for 4 h at room temperature with 100 nM 125 I-CaM in the presence of 0.1% β -lactoglobulin,

washed four times for 2.5 min with buffer C and autoradiographed. (c) For 45 Ca binding to CaN subunit B (CaN B) after transfer to the same nitrocellulose filters the method of Maruyama et al. (39) was used with the following modifications. The proteins were transferred at 200 mA for 18 h and the nitrocellulose filters were washed four times, for 15 min each, with the wash buffer described in reference 39. After exposure to 45 Ca the filters were rinsed twice for 2 min in 100 ml 50% vol/vol ethanol water instead of once with water and once with 50% ethanol.

For these tests bovine testis CaM was iodinated to a specific activity of 70 μ Ci/nmol (1.8 mol iodine/mol) as described by Klee et al. (30). 45 Ca (specific activity, 21.9 mCi/mg) was obtained from New England Nuclear. Polyclonal antibodies against the two subunits of CaN were raised in New Zealand rabbits using 0.3 mg bovine brain CaN mixed with an equal volume of complete Freund's adjuvant. The rabbits were boosted 30 d later with the same amount of protein with incomplete adjuvant and blood was collected a week later. The IgG fraction was prepared by three successive ammonium sulfate precipitations (0–40% saturation at 0°C) of the antisera. The anti-CaN IgG (20 μ g protein/ml) did not recognize proteins other than the two subunits of CaN in Western blots of crude brain extracts to which 7.5 μ g protein were applied. Under the same conditions the lower limit of detection was 5 ng CaN (M. H. Krinks, A. S. Manalan, and C. B. Klee, manuscript in preparation).

Results

Trigger Experiments In Vivo by Microinjections

As Fig. 1 shows, microinjected alkaline phosphatase (0.1 mg/ml) produces massive release of trichocysts (about two-thirds of the number present). (All concentrations indicated are estimated final concentrations in the cell.) This starts after only ~30 s (probably the time required for the enzyme

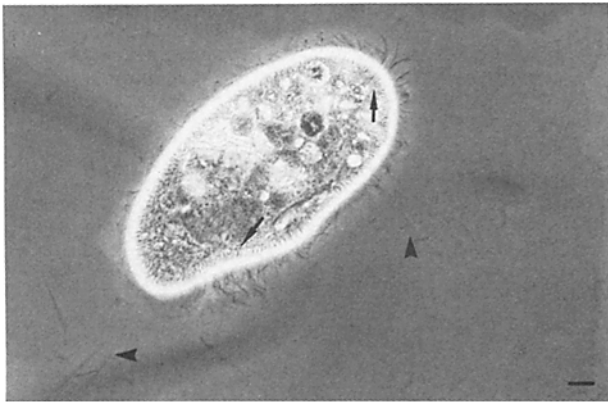


Figure 2. Wild-type (7S) cell 2 min after injection of 10^{-5} M Ca^{2+} in injection buffer. Arrows point to trichocysts still abundantly present in the cell cortex. Only a few trichocysts had been released (arrowheads). Bar, 10 μm .

to get access to the fusion sites) and is terminated after ~ 2 min. Apparently wild-type cells release most of the trichocysts they have docked on the cell membrane. In contrast, strain *nd9-28°C* (nonpermissive) does not release any trichocysts when alkaline phosphatase is injected (Fig. 1, inset).

Microinjections of either injection buffer, CaM, CaN, or Ca^{2+} alone (in concentrations of 80 or 100 $\mu\text{g}/\text{ml}$ or 10^{-5} M,

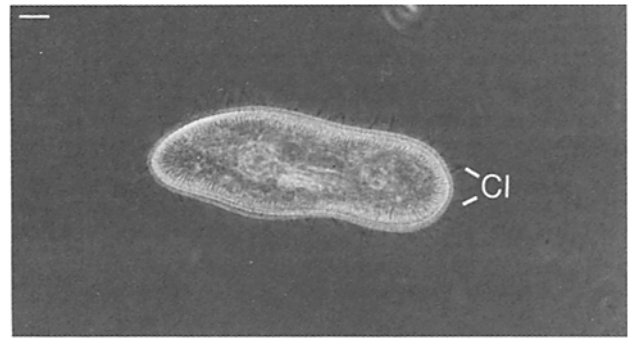


Figure 4. Wild-type (7S) cell microinjected with anti-CaN Ab (0.1 mg/ml final concentration) and exposed to the exocytosis trigger agent AED added to the outside medium (0.005% wt/vol). Exocytosis is totally blocked, as can be seen from the presence of trichocysts (5- μm -long rodlike structures) all over the cell cortex. CI, cilia. Bar, 10 μm .

respectively) into wild-type cells do not produce the release of any substantial number of trichocysts. (Since these controls were so monotonously negative, we present only a micrograph for Ca^{2+} in Fig. 2.) This is particularly surprising for Ca^{2+} , since it is well established that trichocyst release is triggered by a Ca^{2+} -influx (40, 52). Our consistently negative results might have been caused by rapid Ca^{2+} sequestration. Only when we injected a complex of all three



Figure 3. Wild-type (7S) cell 2 min after simultaneous injection of CaN (100 $\mu\text{g}/\text{ml}$ final concentration), CaM (80 $\mu\text{g}/\text{ml}$), and CaCl_2 (10^{-5} M) and a cocktail of protease inhibitors. It was shown in parallel that either component injected alone, even Ca^{2+} , did not induce exocytosis of trichocysts, whereas this experiment revealed massive exocytosis. Bar, 10 μm .

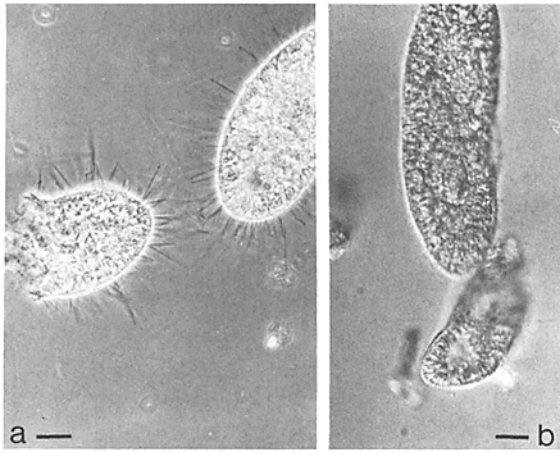


Figure 5. Isolated cortex fragments prepared from wild-type cells (7S) by gentle homogenization in a medium consisting of 5 mM Tris-maleate buffer, pH 7.0, with 10 mM MgCl₂ and 0.01 mM free CaCl₂ present. Exocytosis was triggered in vitro. This causes membrane fusion and elongation (decondensation) of trichocyst contents. The amount of these partly released trichocysts can easily be counted when samples are analyzed in different focal planes. (a) Triggered state (in vitro exocytosis); (b) same preparation but in the presence of anti-CaM Ab which inhibits membrane fusion. Bar, 10 μm.

components, Ca²⁺-CaM-CaN, could we induce massive exocytosis of trichocysts (again about two-thirds of the number present). Again this response started ~30 s after injection and was terminated after ~2 min (Fig. 3). The same result was achieved when a protease inhibitor cocktail was coinjected, as in Fig. 3. Again, injection of Ca²⁺-CaM-CaN into *nd9-28°C* cells remained without any effect. (Cells were observed for up to 18 h, since repair experiments with ciliary mutations required many hours [20].)

In alternative experiments we injected Ab to CaM or to CaN. As shown in the next paragraph the amount of Ab (0.1 mg/ml) that can be injected did not suffice to bind a substantial amount of CaM. The opposite holds true for anti-CaN Ab, which in wild-type cells fully inhibited exocytosis in response to AED (Fig. 4), a potent trigger agent for trichocyst release (50, 52).

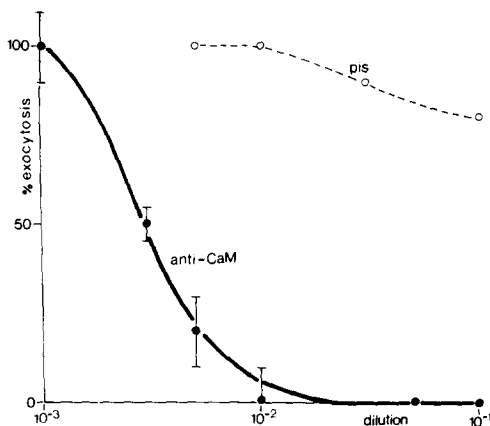


Figure 6. Inhibition of exocytosis in vitro (cortex preparations as in Fig. 5) in response to anti-CaM Ab or to preimmune serum (pis). 10⁻² × dilution = 0.10 mg/ml. Vertical bars, standard error determined for all cortices analyzed.

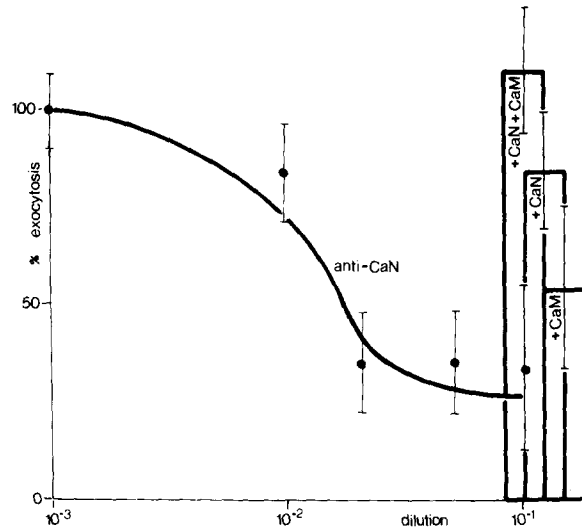


Figure 7. With isolated cortex preparations (see Fig. 5) exocytosis can be inhibited by anti-CaN Ab. 10⁻² × dilution = 0.08 mg/ml. Bars, standard error determined for all cortices analyzed. This effect can be reversed by an excess of CaN (0.1 mg/ml), particularly when CaM (0.08 mg/ml) is also added.

Other Experiments with Whole Cells

We tested the effect of heparin, an acidic compound known to inhibit PP phosphatase activity (17), on AED-triggered exocytosis. For the same reason we also tested polyglutamate and polyaspartate. We found all these compounds to block AED-mediated exocytosis (Table I) when added to the outside medium. Values of inhibitory concentrations (IC₅₀ and IC₁₀₀) are even lower for GLU_n and particularly for ASP_n, when compared with heparin. This aspect is also supported by the failure of protein kinase inhibitors, like diazepam (13) or alloxan (46), to inhibit AED-mediated exocytosis (data not shown).

Results with Isolated Cell Cortices

Cortex preparations have been characterized before (29, 43, 65). Fig. 5 documents that they contain trichocysts still attached to the cell surface and that they are exocytosed when Mg²⁺ is reduced relative to Ca²⁺; it also shows that this effect is inhibited by anti-CaM Ab. This can be quantified as in Fig. 6.

Preimmune sera were not inhibitory (Fig. 6) and practically the same negative results were obtained in another set of controls using nonspecific IgG (anti-ovalbumin, ≤1 mg/ml; data not shown). The inhibitory effect of anti-CaN Ab is documented in Fig. 7; it could be counterbalanced by an excess of CaN, particularly when combined with CaM.

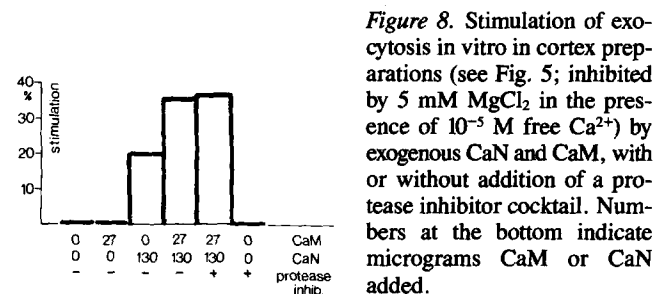


Figure 8. Stimulation of exocytosis in vitro in cortex preparations (see Fig. 5); inhibited by 5 mM MgCl₂ in the presence of 10⁻⁵ M free Ca²⁺ by exogenous CaN and CaM, with or without addition of a protease inhibitor cocktail. Numbers at the bottom indicate micrograms CaM or CaN added.

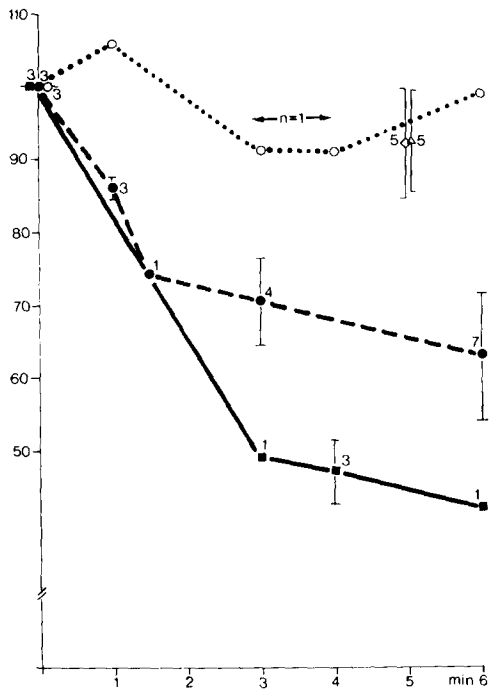


Figure 9. Percentage of dephosphorylation of the 65-kD PP in cortex preparations during exocytosis in vitro (which is completed within ~ 2 min), triggered by adding alkaline phosphatase (dashed line) or a complex of Ca^{2+} -CaM-CaN (solid line); see Materials and Methods. (Abscissa) Time during which cortex preparations were exposed to the enzymes or to buffer (dotted line); 10^{-5} M Ca^{2+} (diamonds); or 10^{-5} M Ca^{2+} + CaM, 27 $\mu\text{g}/\text{ml}$ (triangles), respectively, in control experiments. (Ordinate) Percent of 65-kD PP dephosphorylation in relation to control values at time 0. Numbers (n) indicate double determinations, bars indicate standard deviations.

Table I summarizes our results obtained with exocytosis inhibition in vitro. As in vivo, protease inhibitors did not interfere with exocytosis in vitro. Alternatively, Fig. 8 documents the stimulation of exocytosis in vitro by CaN, notably when combined with CaM, in the presence of 10^{-5} M Ca^{2+} . Again, protease inhibitors remained without any effect on exocytosis performance.

Cortex preparations were the only way to selectively monitor the occurrence of a significant dephosphorylation of the 65-kD PP (according to data obtained from ^{32}P -prelabeled cells) in response to alkaline phosphatase or Ca^{2+} -CaM-CaN (Fig. 9). These experiments also imply the first demonstration of the association of the 65-kD PP with the cell surface complex.

Endogenous CaM and CaN

CaM had been identified before in paramecia according to molecular (53), biochemical (66), and immunological (37, 43, 55) criteria. RIA tests revealed that CaM accounts for 1.5% of cellular protein. In contrast, the occurrence of CaN in these cells has not yet been established in the literature. We found with RIA tests that 0.15% of cellular protein bound anti-CaN Ab, though this might not directly indicate the true CaN concentration in paramecia. (For the reactivity of anti-[bovine brain]CaN Ab with *Paramecium* CaN, however, see below.) From the intracellular concentrations determined for CaM or CaN binding sites, respectively, one could easily

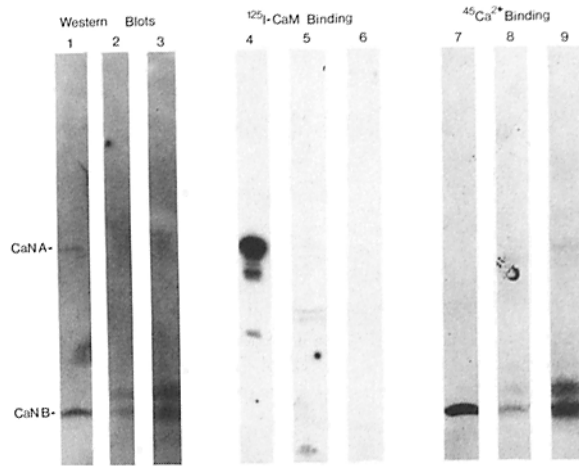


Figure 10. Identification of CaN in *Paramecium*. *Paramecium* cells (lanes 2, 5, and 8) or isolated cortices (lanes 3, 6, and 9) were solubilized in SDS and aliquots of the protein solutions were subjected to SDS-PAGE. Lanes 2 and 3 contained 20 μg protein and were analyzed by Western blots; lanes 8 and 9 contained 60 μg protein and were analyzed for calcium binding; lanes 5 and 6 (120 μg protein/lane) were tested for CaM binding. Lanes 1, 4, and 7 contained 10, 0.5, and 1,000 ng CaN, respectively.

calculate that the amount of Ab (the maximum one can reasonably inject) can bind only little of the CaM present in a cell (see above).

As shown in Fig. 10, *Paramecium*, whole cells, or isolated cortices contain a 15-kD polypeptide which is recognized by anti-CaN IgG. No cross-reacting polypeptides were detected when the gels were treated with control IgG (data not shown). Like CaN B the 15-kD peptide bound calcium after transfer to nitrocellulose filters (Fig. 10, lanes 8 and 9). Thus, on the basis of immunoreactivity, migration in SDS gels, and ability to bind calcium, a CaN B-like protein has been detected in *Paramecium* cells or in isolated cortices. Western blots (Fig. 10, lanes 1-3) revealed only little CaN A-like protein in cortices (lane 3) and none in *Paramecium* cell homogenates. Furthermore, we failed to find a CaN A-like protein by CaM binding assays (Fig. 10, lanes 5 and 6). Failure to detect CaN A by this method might have been due to the existence of tissue- and species-specific isoforms of the catalytic unit of CaN. For instance, the Ab to the bovine brain protein used in these studies failed to recognize several CaN A-like proteins in rabbit or rat tissue extracts other than brain or skeletal muscle (33). The lack of CaM binding may also reflect species specificity, since mammalian instead of *Paramecium* CaM was used for this assay. CaN A is extremely susceptible to proteolysis. Therefore, the failure to detect it in the cell extracts may have been due to its degradation by endogenous proteases. Since in brain extract CaN B is always found associated with CaN A (M. H. Krinks, A. S. Manalan, and C. B. Klee, manuscript in preparation), it is reasonable to assume that a homologue of CaN A is present in *Paramecium* cells. This might be represented by the faint 60-kD band present in Western blots from cortices (Fig. 10, lane 3).

Results obtained with cortex preparations (Fig. 10, lanes 3 and 9) imply, therefore, the occurrence of a CaN-like protein in the cortex of these cells. This holds also for CaM (43) and for the 65-kD PP (Fig. 9).

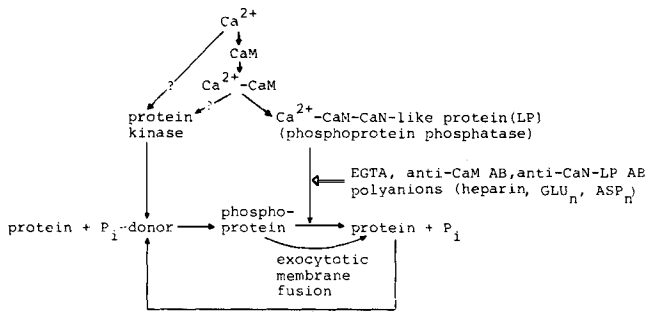


Figure 11. Possible mode of exocytosis induction with the involvement of the 65-kD PP, of CaM and of CaN, as derived from the data presented here. Open arrow indicates inhibition.

Discussion

The goal of this study was to find out whether a PP phosphatase could ignite exocytotic membrane fusion in *Paramecium* cells. This had been expected because of the occurrence of rapid dephosphorylation of a 65-kD PP in response to widely different trigger agents (18, 69). Our own study (69) had shown a strict dependence of the percentage of 65-kD PP dephosphorylation on the concentration of trigger (AED) or inhibitor (neomycin) agents used or on the amount of trichocysts actually released from different mutations or from phenocopies thereof. Yet, theoretically, the dephosphorylation of the 65-kD PP could also have represented an epiphenomenon superimposed on exocytosis induction. We can now derive from our current experiments that PP dephosphorylation via Ca^{2+} -CaM-CaN is in fact directly involved in the induction of exocytotic membrane fusion (Fig. 11).

Evaluation of the Methodical Approach

Could all the trigger effects achieved *in vivo* and *in vitro* with exogenous phosphatases possibly be due to contaminants? (See introduction for possible fusogenic mechanisms discussed in the literature.) We exclude this for the following reasons. (a) Only highly purified preparations were used. (b) Microinjection of either injection buffer, Ca^{2+} , or CaM alone had no effect (Fig. 2). (c) Experiments involving microinjections or exocytosis *in vitro* were occasionally performed with protease inhibitors added (Fig. 3, Table I), but results were not affected. (d) Microinjected protease inhibitors also had no effect on AED-triggered exocytosis. (e) In pilot experiments microinjected proteases did not trigger exocytosis; the only effect was that some (or phospholipase C), in concentrations exceeding the possible level of contaminants, impaired the cells that caused the release of some trichocysts due to visible cell damage. (f) AED-induced synchronous exocytosis had been shown to cause no change of protein patterns, at least in one-dimensional electropherograms (69). (g) Finally, the inhibitory effect of Ab to CaM or CaN most strongly argues against unspecific effects.

Microinjections as used here have proved very useful before, as when Lefort et al. (34) could repair the *nd9-28°C* mutation by a transfer of wild-type cytoplasm. Since we were not able to repair *nd9-28°C* cells by Ca^{2+} -CaM-CaN injections, the repair mechanism, and, hence, the mechanism of membrane fusion, must be more complex, e.g., involving still other components unknown to us so far; this complexity might include certain binding proteins, e.g., for CaM, etc.

Such a defect would then entail a defective assembly of structural components at preformed exocytosis sites. Concomitant ultrastructural defects have indeed been observed when *nd* mutations were analyzed with the electron microscope (51).

Isolated cortex preparations also proved appropriate for testing different requirements for exocytosis. We used them only for a limited time period so that leakage of exocytosis-relevant proteins was minimized. Also, by adding CaM and/or CaN we could considerably increase the exocytosis rate in our experiments. Problems arising from the limited amount of injectable protein, e.g., anti-CaM Ab, could easily be overcome by using these isolated cortex fragments.

Conclusiveness of the Results Obtained

We managed to trigger exocytosis by injecting two widely different phosphatases (Figs. 1 and 3). Unspecific reactions due to cell damage can be excluded on the basis of results obtained in control experiments (buffer, Ca^{2+} , and CaM injection, Fig. 2; *nd9-28°C* cells, Fig. 1, inset) and because of the inhibitory effect of anti-CaN Ab on exocytosis in wild-type cells, in which, alternatively, CaN combined with CaM and Ca^{2+} did trigger exocytosis (Fig. 3).

Though CaM is known to occur in *Paramecium* (37, 43, 53, 55, 66), there have been no data available on the occurrence of CaN-like protein(s) except for some hints from immunohistochemical work (32). We have now filled this gap (Fig. 10), though for an unequivocal demonstration of the CaN A subunit more work would be required. Also we still do not know precisely the Ab-binding capacity of the *Paramecium* antigen(s).

CaM was found in concentrations of 1.5%; this amounted to only slightly more than reported by Satir et al. (55) and may well account for the high recovery (1% of cell protein) obtained by Rauh and Nelson (53). This also explains our failure to inhibit AED-triggered exocytosis *in vivo* by injection of anti-CaM Ab in the usual final concentration of 0.1 mg/ml.

The probable involvement of a Ca^{2+} -CaM-CaN complex in exocytosis induction via dephosphorylation of the 65-kD PP can also be inferred from experiments with isolated cortices (Figs. 5–9). For these preparations Vilmart-Seuwen et al. (65) have documented that the membranes of trichocysts attached to such cortices fuse perfectly with the cell membrane and that trichocyst ghosts do not reseal. Inhibition by anti-CaM Ab was expected, because we had already demonstrated the presence of CaM at preformed exocytosis sites (43). (Similarly, anti-CaM Ab inhibited exocytosis in chromaffin cells [28] and sea urchin eggs [60].) The effect of anti-CaN Ab *in vitro* (Fig. 7) paralleled its effect *in vivo*. It was important to show that the 65-kD PP was efficiently dephosphorylated by any of the phosphatases applied to cortex preparations (Fig. 9).

As stated above, cortices release trichocysts when Mg^{2+} is reduced relative to Ca^{2+} and they react much more efficiently when protein leakage is minimized or when CaM and/or CaN are added in excess (Fig. 8). Leakage or degradation with time might also explain the observed reduction of the exocytotic response of egg cell cortex preparations (61). The ionic conditions we chose for our *in vitro* system appear feasible, since CaN has optimal activity with 10^{-6} M Ca^{2+} with little interference of millimolar Mg^{2+} (68).

In the scheme of Fig. 11 we, therefore, assume the occur-

rence of a Ca^{2+} - and calmodulin-stimulated PP phosphatase which would resemble or be identical to CaN (30, 38). In the course of exocytosis triggering the phosphatase would be activated as soon as intracellular free Ca^{2+} concentration increases (52). As to the kinase which should be postulated (see Fig. 11) we have no information so far on its molecular identity. Yet the failure of protein kinase inhibitors diazepam (13) or alloxan (46) to inhibit exocytosis (data not shown) indicates that the kinase is not responsible for initiating membrane fusion. Since proteolytic cleavage (27, 36, 44) as well as phospholipase activity (42) had also been discussed as a possible fusogenic mechanism, we excluded this aspect in adequate controls (see Results). Applying metalloendoprotease inhibitors to chromaffin cells, Harris et al. (23) also recently obtained negative results.

Comparison with Other Systems

When protein phosphorylation had been analyzed during stimulated secretion in other systems, results were mostly the opposite, i.e., several protein bands were phosphorylated on autoradiograms (2, 6–9, 12, 13, 15, 45, 54, 56, 59, 63). Only occasionally was one or the other band found to be dephosphorylated (2, 10, 11, 59). However, in *Paramecium* the selective dephosphorylation is well established (18, 69).

In principle this discrepancy could have different reasons.

(a) Dephosphorylation can entail selective phosphorylation on autoradiograms when rephosphorylation takes place in the presence of ^{32}P . (b) Phosphorylation might precede actual membrane fusion when ion channels or receptors for secretagogues are involved (14, 24, 45). No such channels or receptors are known for polyamines, like AED, as secretagogues, which are assumed to increase Ca^{2+} -conductivity of membrane lipids (16, 52). (c) Phosphorylation might occur only after membrane fusion, i.e., as a secondary phenomenon. (d) Finally, one could question whether the mechanisms of fusion are the same in different exocytosis systems.

As to these different aspects it is difficult to find out from the published data whether overlapping de- and rephosphorylation could sometimes account for the discrepancies between different systems. It has been claimed that in mast cells a phosphorylation step might terminate, rather than induce, exocytosis (67). It has been found more conclusively that in chromaffin cells irreversible protein phosphorylation by γ -thio-ATP (19) totally blocks exocytosis (4, 5). The phosphorylation of receptors and ion channels has been shown to be crucial for exocytosis in some cells (14, 24, 45), yet this does not appear to be directly relevant to the membrane fusion step.

Though microinjected protein kinase was found to increase neurotransmitter release (35, 45), it has been localized immunocytochemically on synaptic vesicle membranes, i.e., remote from the cell membrane (41). Thus, it could account for effects other than membrane fusion. (Undoubtedly, the occurrence of several dozens of PP in neurons [45] also makes it even more difficult to pinpoint their respective functions.) In contrast, trichocysts of *Paramecium* are closely attached to the cell membrane and the only step to be induced is membrane fusion (46).

Other difficulties are the existence of a number of protein phosphatases, even in one and the same cell type (25, 57), the absence of specific inhibitor drugs, and the broad substrate specificity, e.g., of CaN-type phosphatases (30, 38,

47–49). We tried to obtain specific effects by applying specific antigens (CaM, CaN) or specific antibodies directed against them. The complementary effects achieved with them in vivo as well as in vitro strongly argue for a physiological role of CaM and CaN in our system. (For exocytosis inhibition by anti-CaM Ab in other systems, see references 28 and 60.) The fact that CaN also splits *p*-nitrophenyl phosphate (47–49) is interesting with regard to our previous cytochemical finding of a *p*-nitrophenylphosphatase at the preformed exocytosis sites of *Paramecium* cells; moreover, this reaction was found only in strains capable of exocytotic membrane fusion (51). All these data now appear fully compatible with each other. Membrane fusion might be induced by hydrophobic perturbation of lipid layers by the 65-kD PP in its dephosphorylated form.

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