

Synchronous Exocytosis in *Paramecium* Cells Involves Very Rapid (≤ 1 s), Reversible Dephosphorylation of a 65-kD Phosphoprotein in Exocytosis-competent Strains

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ABSTRACT Synchronous exocytosis in *Paramecium* cells involves the rapid (≤ 1 s) dephosphorylation of a 65-kD phosphoprotein, which, after a lag phase of ~ 5 s, is reversed within ~ 20 s. Exocytosis inhibitors suppress this reaction; stimulatory and inhibitory effects are dose dependent. The dephosphorylation of the 65-kD phosphoprotein occurs only in exocytosis-competent strains, but not in mutant strains that cannot carry out membrane fusion, or that are devoid of secretory organelles or cannot transport them to the cell membrane. Since under all conditions analyzed the transient dephosphorylation of the 65-kD phosphoprotein strictly parallels the actual amount of exocytosed organelles, this process might be involved in exocytosis performance, perhaps in its initiation.

Protein phosphorylation and dephosphorylation processes are now considered to represent a general mechanism for a transient activation of various cell functions (18, 19, 23, 24, 38). Protein phosphorylation accompanies exocytotic activity in endocrine (1, 7, 8, 16, 37) and exocrine (3, 9, 27, 36) gland cells as well as in mast (39, 41) and nerve (11, 23, 25) cells. Whereas a dephosphorylation of single proteins was reported to occur only rarely during stimulation (3, 9, 40), in a comparison of different systems the opposite was observed quite frequently with a considerable number of proteins, which were all of different molecular weights. However, because most systems do not allow one to induce exocytosis in a synchronous way that would definitely exclude possible overlaps of phosphorylation/dephosphorylation processes, their possible role in stimulus–secretion coupling remains largely unknown.

In this respect *Paramecium* cells offer a unique advantage, since by exocytosis they can expel most of their secretory organelles (trichocysts), more than 1,000 per cell, when triggered by certain polyamino compounds (29, 31). The actual time required for exocytosis in one cell, as determined by electrophysiological membrane capacitance changes, is only 1 s.¹ In cell suspensions all processes are accomplished within a few seconds (14). This short reaction time is possible due to the fact that >90% of trichocysts are docked onto the cell membrane, ready for immediate exocytosis (28). This allowed

us to analyze phosphorylation/dephosphorylation cycles under synchronous conditions. Although the precise mode of action of polyamines is not yet known, it may be due to direct effects on protein kinase and phosphoprotein phosphatase activity, as shown with other systems (2, 21, 35). Our analyses clearly show that dephosphorylation may be a crucial step in exocytosis performance.

When we began our study Gilligan and Satir (13) had recently found the dephosphorylation of a 65-kD phosphoprotein in *Paramecium* cells, which discharged trichocysts in response in picric acid; however, because this does not allow the cells to survive, no time sequence or dose-response analyses could be made. With polyamines, however, kinetic analyses are possible and cells can be repeatedly triggered when they are allowed to replenish their secretory stores (26, 29). This permitted us to produce situations with varying amounts of dischargeable trichocysts.

Another unique advantage of the *Paramecium* system is the availability of a battery of mutations (10). Among them are different nondischarge mutations (with trichocysts docked to the cell membrane, or without membrane fusion capacity [5, 20]), mutants characterized by the presence of a small (*tam38*) or large number (*ftA*) of defective free trichocysts in the cytoplasm or by the total absence of trichocysts (*tl*).

With the methodology used here, orthophosphate (³²P_i) labeling, polyacrylamide gel electrophoresis, and quantitative analysis, we obtained, under all conditions analyzed, a strict

¹ Deitmer, J., and H. Plattner. Manuscript in preparation.

correlation between the degree of dephosphorylation of a 65-kD phosphoprotein and the number of trichocysts released. Dephosphorylation is so rapid that it could be overlooked if the system did not work in a synchronous fashion. Thus, we could not only confirm the essential aspects of the previous work by Gilligan and Satir (13) but also add important new information.

MATERIALS AND METHODS

Cell Cultures: *Paramecium tetraurelia* strains used were *K401* and *7S*, the wild types of the following strains: nondischarge mutations *nd6*, *nd7*, and *nd9* (exocytosis inhibited after cultivation at 28°C; normal exocytosis when grown at 18°C [5, 20, 29, 33]); football (*ftA*) with many abnormal, free trichocysts in the cytoplasm (34); *tam38* (with only a few free abnormal trichocysts in the cytoplasm [20]); and trichless (*tl* [34]). In addition, the ciliary mutation *d4-500r*, which lacks functional ciliary Ca²⁺ channels (15), was used.

Since the same results were obtained with *7S* cells grown in a sterile medium according to Kaneshiro et al. (17) as with monoxenically (with *Enterobacter aerogenes* added) grown and extensively starved cells, we cultivated most strains monoxenically in a lettuce medium up to the early stationary phase at 25°C, except for *nd9*, which was grown over five cycles at 18 or 28°C, respectively. We ascertained by light microscopy that food bacteria disappeared when cultures were washed and starved as follows. Cells were first concentrated 10 times and transferred for 4 h to phosphate-free PIPES buffer (5 mM PIPES/HCl + 1 mM CaCl₂ + 1 mM KCl, pH 7.0) in phosphate-free glassware; sterile cultures were treated this way for 24 h.

In Vivo Labeling with ³²P_i and Exocytosis Triggering: Starved cells were further concentrated by filtration (using a tube with a sieve plate with 40-μm pores) to a final density of 5 × 10⁵ cells/ml. To 2.0 ml cells was added 300 μCi of carrier-free ³²P_i from New England Nuclear (Braunschweig, Federal Republic of Germany). After 4 h at 25°C, 100-μl aliquots were drawn into an Eppendorf pipette tip which had been filled with 15 μl of a 0.05 (wt/vol)% solution of the trigger compound aminoethyl-dextran (AED),² which was prepared as before (29, 31); for controls we used 15 μl PIPES buffer instead. This allowed us to obtain very short trigger periods (1 s) when triggered cells were immediately blown into 600 μl boiling sample buffer. Eventually, AED and the exocytosis inhibitor neomycin (Sigma Chemical Co., St. Louis, MO; see reference 29) were used in varying concentrations to obtain dose-response data. Similarly, Mg²⁺ (13) or EGTA (13, 29) were applied as inhibitors.

SDS PAGE, Autoradiography, and Liquid Scintillation Counting: Samples of cells and expelled trichocysts were boiled for 3 min in sample buffer composed of 100 mM Tris/HCl pH 6.8, 5% SDS, 10% glycerol, 5% mercaptoethanol, and 0.01% bromphenol blue as a tracking dye. Usually we applied 100 μg protein (including molecular weight standards) on 2-mm thick slab gels, consisting of a 5% stacking and a 10% resolving acrylamide gel. Gels were run in a Laemmli type system with 15 mA for ~15 h; the 46 V (at the start) was allowed to increase steadily to 169 V.

The protein content per sample was determined by the Lowry standard procedure after precipitation with an equal volume of 15% trichloroacetic acid (2 h, 4°C), centrifugation, and solubilization in 0.4 N NaOH (2 h, 22°C).

Gels were stained with 1% Coomassie Brilliant Blue R250 in 50% methanol plus 10% acetic acid and destained in 25% methanol plus 10% acetic acid. Densitometric evaluation was done with a Desaga densitometer-type Quick Scan at 525 nm. Gels were photographed with an Ilford Pan F film, 18 Din (Ilford Ltd., Basildon, Essex, England) before being dried on a gel slab drier (Bio-Rad Laboratories, Richmond, CA).

Dried gels were subjected to autoradiography for 4–14 d at –70°C after being covered with a Kodak X-Omat AR film in a Kodak X-Omatic cassette with intensifying screens. The AR films were also screened in the densitometer, and the peak area referred to the amount of protein present in the 65-kD band region. Alternatively, this band was cut out from parallel gels (using autoradiography gels as a guide); they were dissolved in 30% H₂O₂ at 60°C for 20 h and supplemented with Beckman Ready-Solv EP scintillation medium (Beckman Instruments Inc., Palo Alto, CA). Liquid scintillation counting was done in a LKB scintillation counter type 1211 Minibeta (LKB Instruments, Inc., Bromma, Sweden). The efficiency was ~6%. Disintegrations per second (dps) referred to the protein content of the respective protein band. All quantitative data thus obtained referred to identical radioactivity values corrected for decay. On the average, data from three experiments were pooled for the radioactivity curves presented.

² Abbreviation used in this paper: AED, aminoethyl-dextran.

RESULTS

Coomassie Blue-stained gels obtained from axenic *7S* cells show many bands (Fig. 1*a*), all of which remain unchanged during exocytosis stimulation by AED. Among these bands only some show up clearly in autoradiograms (Fig. 1*b*), which reveal major bands at 26, 39, 43, 48, 50, and 65 kD, and minor ones at 80, 92, 96, and 110 kD. The 65-kD band is the only one that changes during AED-triggered exocytosis (Fig. 1*b*). Even 1 s (the minimum time we could resolve) after AED is added the intensity of this band is considerably reduced; its intensity increases after 5 s and rephosphorylation is completed after ≥10 s. The original decrease of the intensity of the 65-kD band is about two-thirds of that in the untriggered state; this corresponds well to the percentage of trichocysts actually released (for precise data see references 14 and 26). Fig. 2 was obtained from scans of the 65-kD band on autoradiograms after the application of different doses of the trigger agent AED for 2 s. The density of the 65-kD band reaches its half-maximal value at approximately the ED₅₀ (50% of the effective dose) value and a minimum at the ED₁₀₀ (100% of the effective dose) value previously determined for AED triggering (29). (Under the trigger conditions used cells can discharge up to about two-thirds of their trichocyst populations.)

Food bacteria disappeared from starved cells (4 h) before ³²P_i labeling was started. In addition, neither the Coomassie Blue staining patterns nor the autoradiography bands showed any variation when we used starved, monoxenically grown cells instead of sterile cultures. We therefore used several strains preferentially as monoxenic cultures after excessive starvation.

The temperature-dependent exocytosis mutation *nd9* can dephosphorylate the 65-kD phosphoprotein only when cultivated at the permissive temperature of 18°C. In time sequence studies it behaves like other exocytosis-competent strains (Fig. 3*a*), whereas aliquots grown at the nonpermissive temperature of 28°C keep the 65-kD phosphoprotein at an unaltered level of phosphorylation when AED is added (Fig. 3*b*). This also holds for the other, non-temperature-dependent nondischarge mutations, *nd6* and *nd7*. Results obtained with strains *ftA*, *tl*, or *tam38* are also quite similar. All of these data are compiled in Table I. They were obtained by the quantitative evaluation of the time-dependent de-/rephosphorylation of the 65-kD phosphoprotein, as indicated in Fig. 4, *a–c*.

In Fig. 4 no difference can be seen between the wild type (*7S*) and the mutant strain *d4-500r*, which is characterized by lack of ciliary Ca²⁺ channels (15) and by normal exocytosis performance (31). Since AED-triggered exocytosis is dependent on extracellular Ca²⁺ (29), these results largely exclude the variation of the 65-kD band being connected with a Ca²⁺-dependent ciliary reversal reaction (22), which takes place in other strains used in this study.

None of the strains we analyzed displayed any phosphoprotein bands different from those found in axenic wild-type (*7S*) cells.

Rephosphorylation of the 65-kD phosphoprotein takes from ~10 s (*7S* monoxenic, *d4-500r*, *nd9-18°C*) to 30 s (*7S* axenic, *K401*); (Fig. 4, *a–c*). The reason for this difference is not known, but it is not strain specific (*7S*) and it also does not depend on the culture temperature (25°C for all strains except *nd9-18°C*).

As shown before (29) the antibiotic neomycin at a concen-

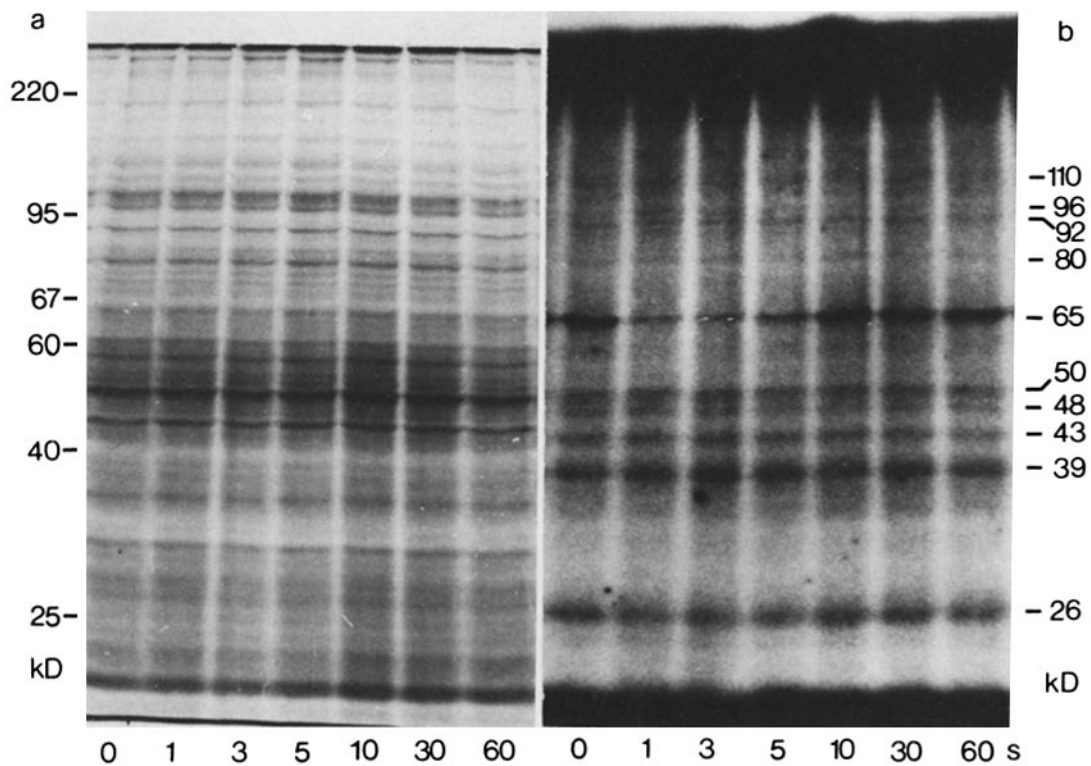


FIGURE 1 Strain 7S (wild type). (a) Coomassie Blue staining patterns at different times after synchronous exocytosis. (b) Corresponding autoradiogram.

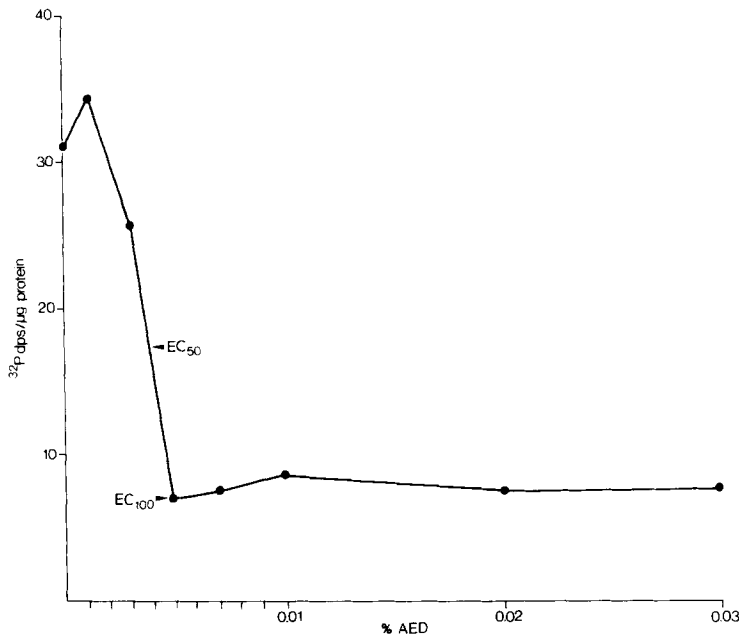


FIGURE 2 Evaluation of scans of the 65-kD band in disintegrations per second per microgram protein (7S cells) obtained 2-3 s after synchronous exocytosis in response to different doses of the trigger agent. EC₁₀₀ and EC₅₀ indicate the concentrations required for maximal and half maximal exocytosis, as determined by counting the numbers of trichocysts actually released (32).

tration of 5×10^{-5} M inhibits AED-triggered exocytosis in *Paramecium* cells. Although the concentration required to inhibit sterilely grown 7S cells (Table II) was somewhat higher than reported before, the inhibition of exocytosis monitored by light microscopy under these conditions paralleled the degree of inhibition of the 65-kD phosphoprotein dephosphorylation. Similarly, Mg^{2+} (as in reference 13) or EGTA suppressed this dephosphorylation step (data not shown) and, simultaneously, exocytosis.

In some experiments we applied a second AED trigger at different times after a previous AED trigger (after which the

cells had been thoroughly washed). The rationale for this approach was as follows. It takes about 9 h or more to replenish the store of dischargeable trichocysts (26). Also, the amount of 65-kD phosphoprotein dephosphorylation should increase as time passes after the first AED trigger. This is indeed what we found (Fig. 5). As one can derive from the data provided by Pape and Plattner (26), the reinsertion of a new set of trichocysts proceeds with a half-time of 3 h. Figs. 5 and 6 (which were obtained by a quantitative evaluation of the 65-kD autoradiography band) reflect these data quite well. This is in agreement with the fact that newly inserted tricho-

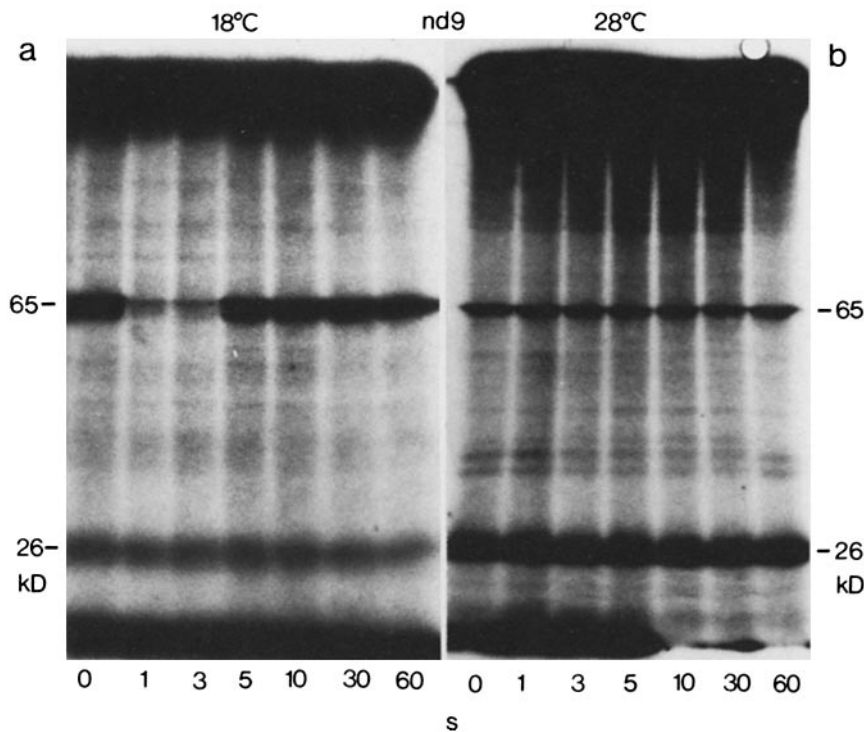


FIGURE 3 Autoradiograms from a time sequence series with *nd9* cells after growing at (a) a permissive and (b) a non permissive temperature.

TABLE I. Relative ^{32}P Labeling Intensity of the 65-kD Phosphoprotein Band in Different *P. tetraurelia* Mutant Strains Before (0 s) and After (1–3 s) AED-triggered Exocytosis*

Strain	Relative labeling intensity		Relative difference b vs. a
	(a) 0 s	(b) 1–3 s	
	%	%	%
7S (axenic)	100*	39	-61
7S (monoxenic)	93	43	-54
K401	86	29	-66
d4-500r	100	50	-50
nd9-18°C	100	43	-57
nd9-28°C	121	125	+3
nd6	107	107	0
nd7	97	97	0
tam38	107	107	0
ftA	157	161	+2
tl	97	97	0

* Data obtained from curves as in Fig. 4 were pooled from density scans and liquid scintillation counting.

* Reference value.

cysts were found to be dischargeable, with only a very short lag period after docking (26).

When one compares the autoradiograms obtained from different strains (Figs. 1 and 3) or from different experimental situations using 7S cells (data not shown), one notices some variability of the 26-kD band. This band may correspond to the 32-kD band, which was noted by Gilligan and Satir (13) to be intensified when paramecia were treated with picric acid in the presence of Mg^{2+} as an exocytosis inhibitor. Under similar conditions, but using AED, we observed the same phenomenon with the 26-kD band. For the following reasons we assume that this band is not relevant to exocytosis. (a) Its intensity did not change with time (after the addition AED) or dose (of AED or of neomycin as an inhibitor). (b) Its intensity was relatively low not only in strain 7S but also in d4-500r, nd6, nd7, nd9-18°C, and tam38, whereas it was high in K401, nd9-28°C, ftA, and tl (where it exceeded the intensity

of the 65-kD band). Therefore, the intensity of the 26-kD band phosphorylation in no circumstances correlates with exocytosis performance. The reason for its variability is not known.

The main result of the present study, which may be derived from Table I (for different genotypes) and from Figs. 5 and 6 (for different phenotypes) is this: The capacity to perform exocytosis does not depend primarily on the relative amount of 65-kD phosphoprotein present in a cell but on the capacity to dephosphorylate this phosphoprotein.

DISCUSSION

We found that exocytosis involves the rapid (≤ 1 s) dephosphorylation of a 65-kD phosphoprotein in all exocytosis-competent *Paramecium* strains. This correlates with the duration of exocytosis in these cells, which is 1 s in an individual cell (according to membrane capacitance measurements¹) or at most a few seconds in a cell suspension (according to morphometric analysis [14]). It is known of this system that exocytosis is independent of transcellular transport of secretory organelles (26), of the participation of microtubules (30), or of microfilament functions (32), and that secretory contents are not phosphorylated (6). The possibility can also be excluded that the decrease of the 65-kD phosphoprotein phosphorylation is due to the ciliary reversal reaction which also occurs when the intracellular free Ca^{2+} concentration increases (22) in response to AED triggering. Evidence for this is derived from results obtained with the ciliary mutation d4-500r (Table I) which are not different from the wild type. Moreover, no significant phosphoprotein of this size can be recognized in ciliary preparations (12).

It would obviously be difficult to catch such a short-lived step, which is immediately counteracted by rephosphorylation within ~ 20 s, if exocytosis were not synchronous. In paramecia, Gilligan and Satir (13) had observed the dephosphorylation of the 65-kD phosphoprotein only when they used a fixative as a trigger agent. At this time most work on phos-

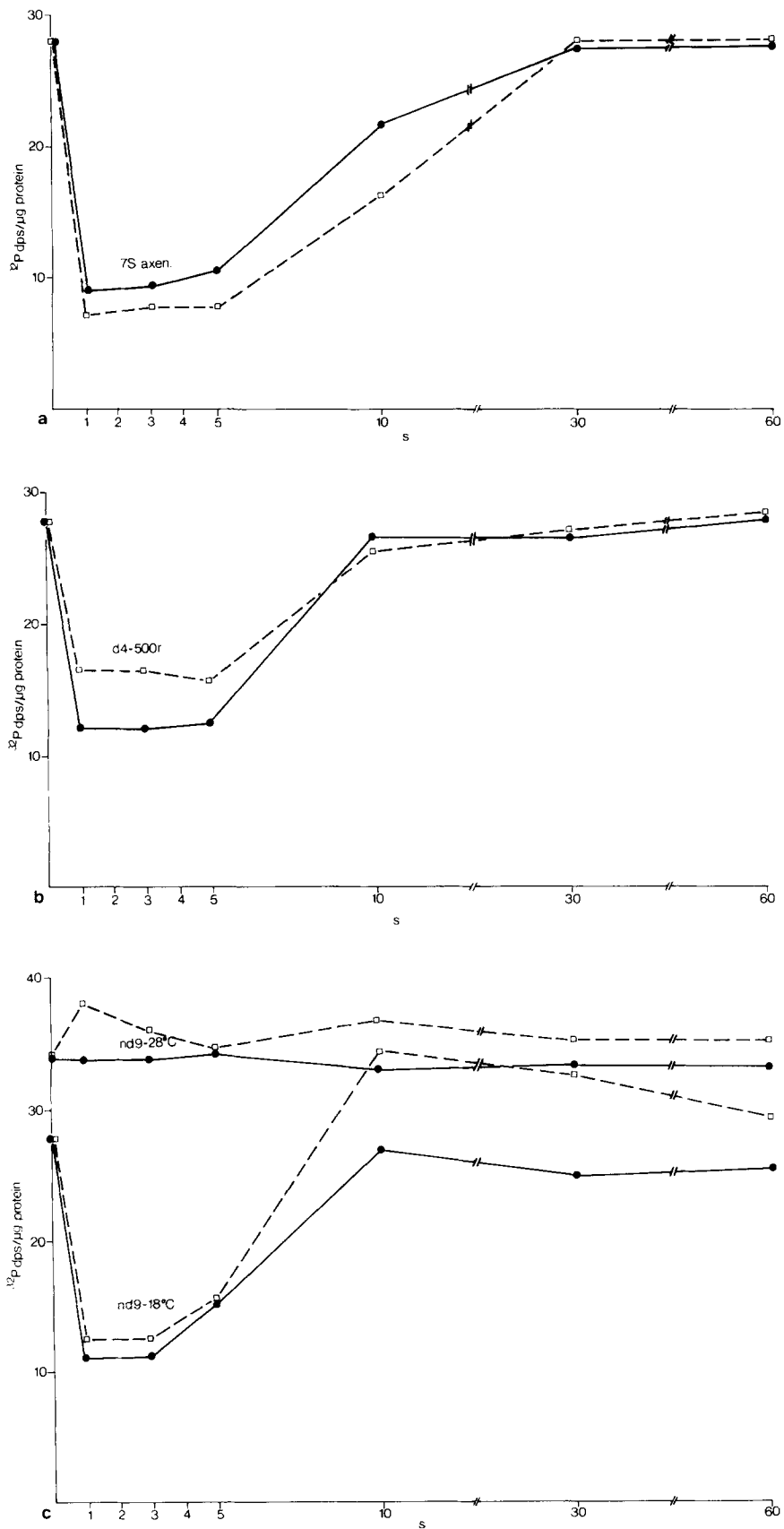


FIGURE 4 Time course of the intensity of radioactive labeling of the 65-kD phosphoprotein band as determined by liquid scintillation counting (units are disintegrations per second per microgram protein; solid line) or from scans of autoradiograms (dashed line) in relative units; data were normalized. Strains analyzed are (a) 7S (axenic), (b) d4-500r, (c) nd9-18°C and nd9-28°C.

phorylation processes in other exocytotic systems can only be done on a time scale of minutes. As in paramecia the size range of proteins phosphorylated under stimulatory conditions is very variable, mostly between 10 and 100 kD, depending on the system, whereas it was noted only rarely that

some endogenous phosphoproteins are dephosphorylated under stimulatory conditions (3, 9, 40). In all cases it was difficult to judge the relevance of these phenomena to the initiation of exocytosis, and so far no common (phospho-)protein has been identified that would regulate exocytosis in different

TABLE II. Inhibition of AED-induced Dephosphorylation of the 65-kD Phosphoprotein by Neomycin (NM)*

	dps/ μg protein*
Control	27.3
Control + 50 μM NM	26.3
AED alone	8.0
AED	
+6 μM NM	7.4
+10 μM NM	14.8
+16 μM NM	22.1
+30 μM NM	22.8
+50 μM NM	27.3

* AED was used at 1.4×10^{-6} M (EC_{100}).

† Refers to the amount of protein present in the 65-kD band.

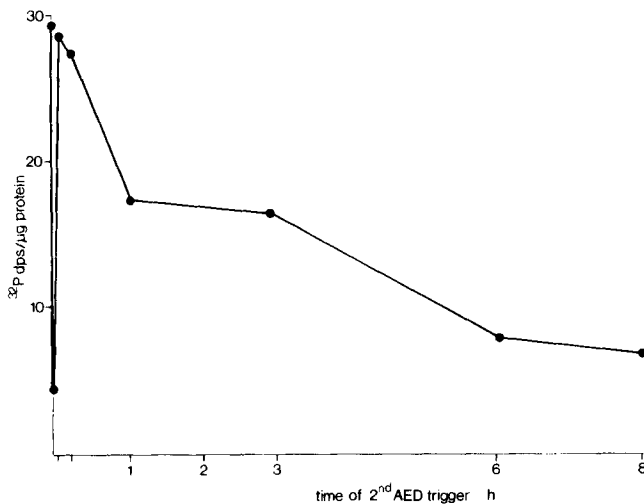


FIGURE 5 Quantitative evaluation (by liquid scintillation counting; units are disintegrations per second per microgram protein) of the 65-kD band under the following conditions (double trigger experiments): 7S cells were first triggered (2-s value) and then exposed to a second trigger at different points after the first trigger. The capacity to dephosphorylate the 65-kD phosphoprotein is slowly re-established to the same extent as new trichocysts become docked to the cell membrane and, thus, available for exocytosis (compare reference 26). After 8 h a situation such as in controls has almost been attained.

systems.

In the *Paramecium* system the availability of a variety of exocytotic mutations offers another considerable advantage. Strains 7S (wild type), *K401*, and *d4-500r*, which can instantaneously expel up to 90–96% of their trichocysts (Table I in reference 29), rapidly dephosphorylate a large fraction of their 65-kD phosphoprotein when triggered by AED (Table I). Strain *nd9* (Fig. 3) also behaves like the wild type (Figs. 1 and 4a) when grown under permissive culture conditions (18°C). When grown at a nonpermissive temperature (28°C), it displays neither exocytosis nor the dephosphorylation of the 65-kD phosphoprotein; this is in agreement with data obtained by Gilligan and Satir (13) with picric acid. As summarized in Table I, we achieved similar results not only with the non-temperature-dependent nondischarge mutations, *nd6* and *nd7*, but also with strains *ftA* and *tam38*, which contain only defective, free (in the cytoplasm), nondischargeable trichocysts or none at all (trichless, *tl*). Table I shows also that in all strains analyzed the 65-kD phosphoprotein is present in comparable amounts and that its dephosphorylation is strictly coupled to actual exocytosis performance. (Only about two-

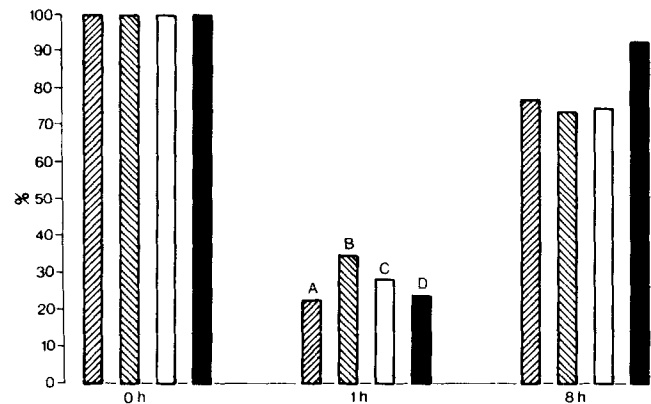


FIGURE 6 Coincidence in 7S cells of the number of (A) potential docking sites on the cell membrane occupied by a trichocyst, (B) rosettes, (C) the number of dischargeable trichocysts, and (D) the capacity to dephosphorylate the 65-kD phosphoprotein. A–C are from reference 26. 0 h indicates normalized data (100%) for controls; 1 and 8 h data are from cells triggered by AED at 0 h and analyzed during reinsertion of new trichocysts 1 or 8 h later by a second AED trigger.

thirds of all trichocysts are released when mass cultures are triggered by AED [14, 26].) Inhibitors of exocytosis such as neomycin or Mg^{2+} also suppress the 65-kD phosphoprotein dephosphorylation (Table II).

Other evidence for a causal connection between the two events comes from double trigger experiments (Fig. 5), in which cells were first depleted of their trichocysts, which they replenished over ≥ 9 h with the half-life of ~ 3 h (26). Again, the degree to which the 65-kD phosphoprotein can be dephosphorylated strictly parallels the number of trichocysts available for exocytosis (Fig. 6) and the number of “fusion rosettes” (see below) formed during this period (26).

Strains that can perform exocytosis are known to display not only “fusion rosettes,” i.e., characteristic aggregates of freeze-fracture particles at potential fusion sites (5, 28), but also an ultrastructurally visible, biochemically not yet identified “connecting material,” probably proteins, between the trichocyst and the cell membrane (33). “Rosettes” and “connecting material” are lacking in different exocytosis-defective mutations (Pouphile, M., M. Lefort-Tran, M. Rossignol, J. Beisson and H. Plattner, manuscript in preparation, and references 4, 5, 20, and 33). The findings reported here may be somehow connected with such ultrastructural features. Moreover, by ultrastructural cytochemistry we were able to localize an ATP- and *p*-nitrophenylphosphate-splitting enzyme activity precisely at the secretory sites, selectively in exocytosis-competent strains (33). This might represent an equivalent of the Ca^{2+} -dependent de-/rephosphorylation sequence reported here. All of these aspects—exocytosis performance (29), dephosphorylation of the 65-kD phosphoprotein (this study), and the formation of the cytochemical reaction product (33)—depend on the presence of exogenous Ca^{2+} , which enters the cell somewhere through the somatic (non-ciliary) membrane (31), possibly just at the secretory sites. The nondischarge mutations used here are affected on different loci (10). Microinjection studies in which trichocysts and/or cytoplasm was transferred from different strains to others revealed that the genetic lesion may reside in the trichocyst or cell membrane (*nd7*, *tam38* [20]) or in a cytoplasmic component (*nd9-28°C* [5]). All of these components appear to cooperate only when properly assembled at the

actual zone of exocytotic membrane fusion, which then displays a characteristic organization (see above). *nd9* cells grown under nonpermissive conditions (5), as well as *nd6* and *nd7* cells, lack not only "rosettes" (20) but also "connecting materials" between the cell membrane and the membrane of the nonextrudable trichocyst attached to the cell membrane (Pouphile, M., M. Lefort-Tran, M. Rossignol, J. Beisson, and H. Plattner, manuscript in preparation). It appears, therefore, that all of these structural elements must definitely be present in a certain spatial arrangement to guarantee normal exocytosis and that this arrangement is a prerequisite for the phenomena reported in this study.

Could the dephosphorylation of the 65-kD phosphoprotein be due to the membrane resealing process immediately after exocytosis? According to ultrastructural observations (14, 32) and electrophysiological data¹ this process is also very fast and synchronous in *Paramecium*. However, since it was found that it probably takes several seconds for ghost membranes to be pinched off the cell membrane in the whole population of cells (14), whereas the dephosphorylation of the 65-kD phosphoprotein requires <1 s (the minimum time we could achieve in this trigger study), this step is much more likely to occur at the beginning rather than at the end of the exocytotic process.

The only other protein with a variable degree of phosphorylation was 26 kD in size. Whereas we can confirm that this is more heavily phosphorylated with Mg^{2+} added as an exocytosis inhibitor (as reported before in reference 13), a similar phenomenon does not occur with neomycin. Moreover, when different mutant strains, exocytosis competent or incompetent, are compared, there is no correlation between the density of this band with the respective exocytotic capacity.

In conclusion, there are different types of evidence to suggest that the dephosphorylation of the 65-kD phosphoprotein may be a crucial step in exocytosis performance in *Paramecium* cells. It might be involved in the initiation of exocytosis, although this hypothesis requires further analysis. The fact that no such phenomenon was observed in other systems may be explained by the very short time this step requires, after which it is rapidly counterbalanced by a rephosphorylation process, once exocytosis has been accomplished. Indeed, to pinpoint the dephosphorylation of the 65-kD phosphoprotein, we had to inactivate synchronously triggered cells very rapidly, i.e., by bringing them to 95°C within 0.2 s (as monitored by thermocouple measurements; data not shown).

Because insufficient pharmacological data are so far available on the *Paramecium* system, it remains to be seen what type of phosphoprotein phosphatase and protein kinase are engaged. The trigger effect of the polyamine secretagogue used in this study might be due to the fact that polyamines not only stimulate phosphatase activity, e.g., in liver (2) and hepatoma cells (21), but also depress protein kinase activity in other systems (35). Since the 40-kD AED used here does not enter the cells, the target structure for its secretagogue function would have to be located on the cell membrane, perhaps precisely at the site where exocytosis occurs.

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