

# A Ca<sup>2+</sup> Influx Associated with Exocytosis is Specifically Abolished in a *Paramecium* Exocytotic Mutant

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**Abstract.** A *Paramecium* possesses secretory organelles called trichocysts which are docked beneath the plasma membrane awaiting an external stimulus that triggers their exocytosis. Membrane fusion is the sole event provoked by the stimulation and can therefore be studied per se. Using 3  $\mu$ M aminoethyl dextran (AED; Plattner, H., H. Matt, H. Kersken, B. Haake, and R. Stürz, 1984. *Exp. Cell Res.* 151:6–13) as a vital secretagogue, we analyzed the movements of calcium (Ca<sup>2+</sup>) during the discharge of trichocysts. We showed that (a) external Ca<sup>2+</sup>, at least at  $3 \times 10^{-7}$  M, is necessary for AED to induce exocytosis; (b) a dramatic and transient influx of Ca<sup>2+</sup> as measured from <sup>45</sup>Ca uptake is induced by AED; (c) this influx is in-

dependent of the well-characterized voltage-operated Ca<sup>2+</sup> channels of the ciliary membranes since it persists in a mutant devoid of these channels; and (d) this influx is specifically abolished in one of the mutants unable to undergo exocytosis, nd12. We propose that the Ca<sup>2+</sup> influx induced by AED reflects an increase in membrane permeability through the opening of novel Ca<sup>2+</sup> channel or the activation of other Ca<sup>2+</sup> transport mechanism in the plasma membrane. The resulting rise in cytosolic Ca<sup>2+</sup> concentration would in turn induce membrane fusion. The mutation nd12 would affect a gene product involved in the control of plasma membrane permeability to Ca<sup>2+</sup>, specifically related to membrane fusion.

**M**EMBRANE fusion, which is the last step of the exocytotic pathway, is a ubiquitous process in eukaryotic cell life, yet the underlying mechanism is almost completely obscure compared to that of other aspects of membrane traffic. Much evidence indicates that the calcium ion (Ca<sup>2+</sup>) plays a key role in regulated exocytosis, as discussed by Douglas (1974) and Knight et al. (1989). However, in most cellular systems studied, the exocytotic process comprises several steps, from protein synthesis and transit to granule targeting toward the plasma membrane and membrane fusion, so that it is difficult to identify the precise step(s) sensitive to Ca<sup>2+</sup> and in particular to know whether the membrane fusion itself is Ca<sup>2+</sup> dependent in vivo.

In *Paramecium*, membrane fusion in exocytosis can be studied per se since the secretory vesicles (trichocysts) are docked at predetermined sites of the cell cortex in a "pre-fusion" state, awaiting an external stimulus that triggers the fusion of their membrane with the plasma membrane (for recent reviews see Plattner, 1987; Adoutte, 1988; Satir et al., 1988). Moreover, synchronous discharge can be triggered by aminoethyl dextran (AED)<sup>1</sup> without damage to the cell (Plattner et al., 1984). Several results argue for the direct involvement of external Ca<sup>2+</sup> in membrane fusion in this organism. (a) External Ca<sup>2+</sup> seems necessary for exocytosis to take place (Matt et al., 1978; Gilligan and Satir, 1983; Garofalo et al., 1983; Garofalo and Satir, 1984) at a concen-

tration of  $10^{-6}$ – $10^{-5}$  M (Plattner et al., 1985). (b) Ca<sup>2+</sup> ionophores in the presence of Ca<sup>2+</sup> can trigger exocytosis (Plattner, 1974; 1976; Matt et al., 1978, 1980; Satir and Oberg, 1978), although the response appears far from complete (see the quantification of Satir and Oberg, 1978). (c) Transient elevation of the concentration of free Ca<sup>2+</sup> in the cytosol ([Ca<sup>2+</sup>]<sub>i</sub>) was shown to be associated with exocytosis (Matt et al., 1978; Plattner et al., 1985). *Paramecium* has been known for a long time to have voltage-operated Ca<sup>2+</sup> channels which are localized on ciliary membranes (for reviews see Kung and Saimi, 1985; Machemer, 1989), however, the external Ca<sup>2+</sup> supposed necessary for exocytosis does not enter the cell via these channels. Indeed, mutants that are devoid of functional voltage-operated Ca<sup>2+</sup> channels, called pw (Satow and Kung, 1980), show normal exocytosis when triggered (Adoutte et al., 1981; Plattner et al., 1984), as do deciliated cells (Plattner et al., 1984). A Ca<sup>2+</sup> channel specific for trichocyst exocytosis therefore may be present in *Paramecium*, as has already been suggested (Satir et al., 1988; Satir, 1989).

Numerous mutants, called nd (for nondischarge), have been isolated that are unable to perform trichocyst exocytosis (Pollack, 1974; Sonneborn, 1974; Nyberg, 1978; Cohen and Beisson, 1980), and they fall into 12 complementation groups (Bonnemain and Cohen, manuscript in preparation). The precise compartment (trichocyst, plasma membrane, cytosol) altered by each of the mutations can be determined by microinjection experiments (Aufderheide, 1978; Beisson

1. Abbreviation used in this paper: AED, aminoethyl dextran.

et al., 1980; Cohen and Beisson, 1980; Lefort-Tran et al., 1981). The physiology of exocytosis can therefore be approached by the analysis of its alterations in the mutants.

For all these reasons, *Paramecium* provides an excellent experimental model for the general problem of  $\text{Ca}^{2+}$  entry during the membrane fusion step of regulated exocytosis. We first reinvestigated the dependence of trichocyst exocytosis on external  $\text{Ca}^{2+}$  and we then studied  $\text{Ca}^{2+}$  fluxes in wild type, pw, and nd mutant cells. Using  $^{45}\text{Ca}$  uptake measurements, we have identified a previously undescribed  $\text{Ca}^{2+}$  influx associated with exocytosis in wild type and pw cells, which can be interpreted as a transient increase in membrane permeability to  $\text{Ca}^{2+}$ . Among the 6 nd mutants tested, one was found to lack the exocytosis-induced  $\text{Ca}^{2+}$  influx. To our knowledge, it is the first time that a mutant with a defect in  $\text{Ca}^{2+}$  permeability, specific for membrane fusion in exocytosis, has been described.

## Materials and Methods

### Strains and Culture Conditions

The wild type strain used in this study was stock d4-2, a derivative of stock 51 of *P. tetraurelia* (Sonneborn, 1975). Mutations belonging to three classes were used. (1) nd6, nd7, nd9<sup>a</sup> (Sonneborn, 1974), nd12 (Cohen and Beisson, 1980), nd169 (Nyberg, 1978), and nd3<sup>c</sup> (Bonnemain, H., and J. Cohen, manuscript in preparation) which affect only the final step of exocytosis (membrane fusion); the nd9<sup>a</sup> mutation is expressed only at temperatures above 24°C (Beisson et al., 1980) and nd12 and 35°C (Cohen and Beisson 1980), just below the limit temperature of 36°C for *Paramecium* growth. The morphology of the exocytotic site of the various nd mutants used here is well known: they all lack the "rosette", an array of particles seen in freeze fracture microscopy, and the subjacent "connecting material", except nd12 which displays a normal exocytotic site (Beisson et al., 1976; Lefort-Tran et al., 1981; Pouphe et al., 1986). (2) tam8 (Beisson and Rossignol, 1975), which blocks trichocyst migration and attachment to the cell cortex and therefore possesses a plasma membrane devoid of attached trichocyst. (3) pWA (d4-500), which prevents ciliary beating reversal upon membrane depolarization (Satow and Kung, 1980) but does not affect exocytosis (see Introduction). The double mutants pWA-nd3<sup>c</sup>, pWA-nd6, pWA-nd7, pWA-nd9<sup>a</sup>, pWA-nd12, and pWA-nd169 were obtained by crossing each nd mutant with the pWA mutant according to the standard method (Sonneborn, 1970) or to a rapid method using 96-well titration plates (Cohen and Beisson, 1980) and looking for recombinants in the F2 progenies. The double mutants associate both of the single mutant phenotypes, i.e., lack of ciliary reversal in depolarizing conditions plus absence of trichocyst exocytosis.

Cells were grown in a grass infusion or wheat grass powder (Pines International, Lawrence, Kansas) infusion bacterized the day before use with *Klebsiella pneumoniae* and supplemented with 0.4  $\mu\text{g}/\text{ml}$   $\beta$ -sitosterol according to Sonneborn (1970). The standard temperature of growth was 26–27°C, unless otherwise specified.

### AED Synthesis

AED was synthesized according to Plattner et al. (1984) and references therein with minor modifications (adaptation of the method was performed with the collaboration of H. Husson and A. Husson, Institut des Substances Naturelles du CNRS, Gif-sur-Yvette): 5.55 g NaOH and 5.55 g 2-aminoethyl hydrogen sulfate (Aldrich Chemical Co., Milwaukee, WI) were dissolved in 11 ml  $\text{H}_2\text{O}$ . 3.66 g of Dextran T40 (Pharmacia, Uppsala, Sweden), dissolved in the mixture, and dried overnight at 105°C. 60 ml of HCl 0.3 N were added for dissolution of the desiccated material and the pH was adjusted to 9.5 with concentrate HCl yielding a brown precipitate which was eliminated by centrifugation. The supernatant was filtered on paper, extensively dialyzed against  $\text{H}_2\text{O}$  until neutralization, and lyophilized. 0.9 g of powder were obtained and dissolved as a 5% stock solution in  $\text{H}_2\text{O}$ . This AED solution proved to be efficient at a final dilution of 1:400, representing  $\sim 3 \mu\text{M}$ , if pure AED. The potency of our AED is a little lower than the one of a sample kindly provided by Dr. Plattner, in that higher concentrations are needed to be efficient.

### Determination of the External $\text{Ca}^{2+}$ Concentration Necessary for Exocytosis

Cells were washed and concentrated by low-speed centrifugation (see below) in 1 mM HEPES, pH 7.1, 0.5 mM  $\text{MgCl}_2$ , 0.5 mM  $\text{CaCl}_2$  and distributed in tubes containing different amounts of EGTA to obtain final concentrations of free  $\text{Ca}^{2+}$  between  $10^{-5}$  and  $10^{-8}$  M (using  $K_{\text{Ca}/\text{EGTA}} = 7.62 \times 10^6$  and  $K_{\text{Mg}/\text{EGTA}} = 5.33 \times 10^4$  as dissociation constants at pH 7.1; Portzehl et al., 1964). The external  $\text{Ca}^{2+}$  concentration was experimentally checked using the fluorescent indicator Quin 2 according to Grynkiewicz et al. (1985). Exocytosis induced by AED prepared in each of the Ca/EGTA buffers was monitored after equilibration of cells for 5–10 min in these Ca/EGTA buffers.

### Determination of Variations in the Intracellular Free $[\text{Ca}^{2+}]$

In *Paramecium* it is possible to monitor internal  $\text{Ca}^{2+}$  concentration by observing behavior. Backward swimming, corresponding to ciliary reversal, indicates that  $[\text{Ca}^{2+}]_i$  is higher than  $6 \times 10^{-7}$  M (Nakaoka et al., 1984) and provides a simple means of detecting an increase in  $[\text{Ca}^{2+}]_i$  above this concentration. Induction of exocytosis always triggers concomitant brief backward swimming (Matt et al., 1978; Plattner et al., 1985) even in pw mutants (Adoutte, 1988), which in other conditions never swim backward as they lack voltage-operated  $\text{Ca}^{2+}$  channels (Satow and Kung, 1980).

Individual cells were collected with a micropipette, washed in 1 mM Tris-HCl, pH 7.4, 1 mM  $\text{CaCl}_2$ , and introduced into an equal volume of 6  $\mu\text{M}$  AED on a slide. The swimming behavior of the cell was observed under a microscope at a low magnification during the stimulation.

### $\text{Ca}^{2+}$ Flux Studies

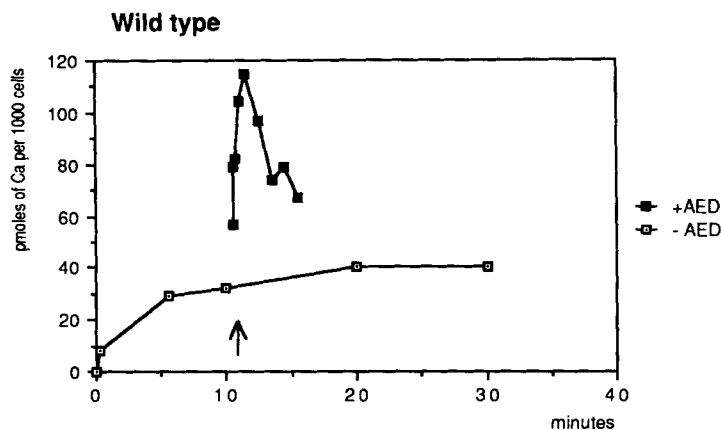
To study the fluxes of  $\text{Ca}^{2+}$  during exocytosis, we developed a method for  $^{45}\text{Ca}$  uptake measurements adapted from Browning and Nelson (1976) and Browning et al. (1976), which, in addition to the results obtained by these authors at 0°C, permitted experiments at 26°C and even 35°C. This protocol was worked out with the helpful advice of M. Claret and B. Berthou. The cells were harvested in log phase (2,000–3,000 cells/ml) by a 5-min low-speed centrifugation (30 g) to avoid unwanted exocytosis before the experiment, washed once in 1 mM HEPES, pH 7.0, 40  $\mu\text{M}$   $\text{CaCl}_2$  (a  $\text{Ca}^{2+}$  concentration low enough to prevent nonspecific binding on cell membranes; Naitoh and Yasumasu, 1967), and concentrated to at least 10,000 cells/ml. After 30 min or more at 26°C (unless otherwise specified) in this incubation buffer,  $^{45}\text{Ca}$  (used at a final specific activity of 1  $\mu\text{Ci}/\text{ml}$ ) uptake experiments were performed. For each point, a 0.5-ml sample of labeled cells was quickly diluted in 4 ml of 1 mM HEPES, pH 7.0, 5 mM  $\text{CaCl}_2$  at 0°C, vacuum filtered over 0.45- $\mu\text{m}$  pore size filters (Millipore Continental Water Systems, Bedford, MA), and washed three times with 4 ml of the same buffer. Each filter was then counted by liquid scintillation. For steady-state flux analysis, basal incorporation of  $^{45}\text{Ca}$  was first recorded and, after 10 min of incubation, 0.5-ml samples of cells were stimulated with AED at a final concentration of 3  $\mu\text{M}$  and kept for various times (a few seconds to a few minutes) before dilution and filtering. To measure the initial rate of uptake,  $^{45}\text{Ca}$  was added to 0.5-ml cell samples with or without AED at the same time and cells were incubated for kinetics of a few seconds before dilution and filtering. Under these conditions, the initial linear phase of  $^{45}\text{Ca}$  uptake represents the  $\text{Ca}^{2+}$  influx through the plasma membrane (Mauger et al., 1984).

## Results

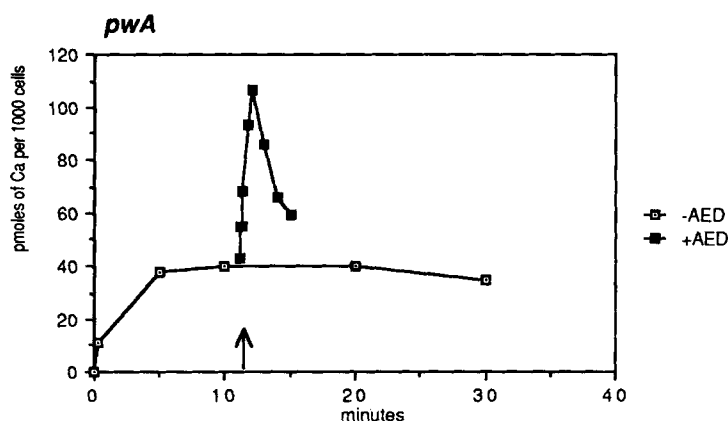
### External $\text{Ca}^{2+}$ Is Necessary for AED-induced Exocytosis

By chelating  $\text{Ca}^{2+}$  with EGTA, Plattner et al. (1985) showed that a concentration of  $10^{-5}$  M free  $[\text{Ca}^{2+}]$  in the external medium was necessary for AED to trigger exocytosis. However, as the authors noticed, low  $[\text{Ca}^{2+}]$  buffers are also quickly lethal for the cells. Repeating this experiment, we obtained the same results and also observed that the cells immediately lyse if excess  $\text{Ca}^{2+}$  is added back to low  $[\text{Ca}^{2+}]$  in-

a



b



**Figure 1.**  $^{45}\text{Ca}$  uptake by wild type (a) and pwa (b) cells upon AED stimulation at the steady state. Cells were incubated for 11 min in  $^{45}\text{Ca}$  before nine 0.5-ml samples were taken, stimulated with AED, and filtered after 5, 10, 15, and 30 s and 1, 2, 3, 4, and 5 min of incubation. A net peak of incorporation above the control level (open squares) is observed when cells are stimulated with AED (black squares). In such experiments, the time constant for basal  $\text{Ca}^{2+}$  exchange, determined as the time necessary to reach 63% (1-1/e) of the plateau, varied from 5 to 10 min, here ~10 min in a and 5 min in b.

cubation media, indicating that cell membranes have been damaged by the treatment.

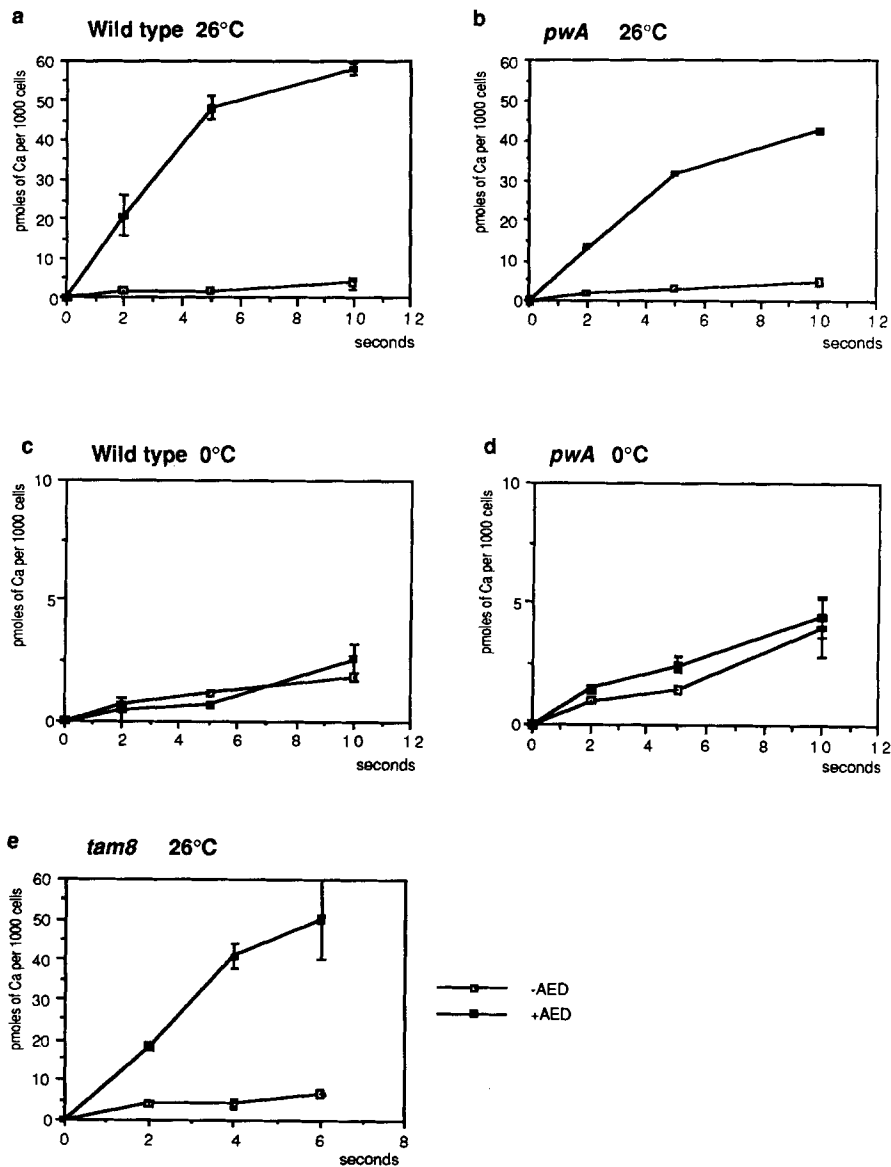
To preserve membrane integrity, we maintained a constant concentration of external divalent cation by adding 0.5 mM  $\text{MgCl}_2$  to the Ca/EGTA buffers. In these conditions, we observed that the cells can survive 15–20 min in  $10^{-8}\text{M}$   $[\text{Ca}^{2+}]$  and up to 1–2 h above  $3 \times 10^{-8}\text{M}$   $[\text{Ca}^{2+}]$ . Cells preincubated in various concentrations of free  $\text{Ca}^{2+}$ , from  $10^{-5}$  to  $10^{-8}\text{M}$ , were submitted to  $3\ \mu\text{M}$  AED, and their trichocyst release was monitored. We observed that AED can trigger massive exocytosis down to  $3 \times 10^{-7}\text{M}$  free  $[\text{Ca}^{2+}]$ , but not at  $10^{-7}\text{M}$  free  $[\text{Ca}^{2+}]$  and below. Therefore, external  $\text{Ca}^{2+}$  seems necessary for AED to trigger exocytosis. One possibility is that AED requires  $\text{Ca}^{2+}$  in order to bind to the membrane and be effective. Alternatively, a transmembranar  $\text{Ca}^{2+}$  gradient and an influx of this ion could be necessary for AED-induced exocytosis. To test this hypothesis,  $\text{Ca}^{2+}$  influx was measured from  $^{45}\text{Ca}$  uptake experiments.

#### *A $\text{Ca}^{2+}$ Influx Is Associated with Exocytosis*

To analyze the  $\text{Ca}^{2+}$  movements associated with exocytosis, we developed a method for  $^{45}\text{Ca}$  measurements based on that of Browning and Nelson (1976) and Browning et al. (1976). In contrast to these results, we were able to observe uptake at room temperature, as also described by Martinac

and Hildebrand (1981), but using simpler methods with better time resolution. Furthermore, to avoid interference with the well-characterized voltage-operated  $\text{Ca}^{2+}$  channels (Kung and Saimi, 1985), we worked in parallel on the pw mutant, devoid of these channels (Satow and Kung, 1980) and wild type cells.

Fig. 1 illustrates an experiment on wild type and pwa cells. Addition of AED initiated a strong increase of  $^{45}\text{Ca}$  uptake by cells. This was transient (~1 min) and followed by a decline of the  $^{45}\text{Ca}$  content to the resting level. These experiments are indicative of a marked increase of  $\text{Ca}^{2+}$  influx initiated by AED in the two cell types, but the results are essentially qualitative. The true  $\text{Ca}^{2+}$  influx initiated by AED must be measured over a period that is quite short compared with the time constant of  $\text{Ca}^{2+}$  exchanges through the plasma membrane (Mauger et al., 1984). In this way, undesirable labeling of intracellular  $\text{Ca}^{2+}$  can be avoided: the influx of  $^{45}\text{Ca}$  is neither altered by  $\text{Ca}^{2+}$  efflux from the cells nor by a redistribution of  $\text{Ca}^{2+}$  into the internal pools in response to the massive AED-mediated uptake of  $\text{Ca}^{2+}$ . Thus,  $\text{Ca}^{2+}$  influx will depend only on the external  $[\text{Ca}^{2+}]$  and on the permeability of the plasma membrane. If external  $[\text{Ca}^{2+}]$  is experimentally fixed, any change in the initial rate provoked by AED will directly reflect the modification of the translocation rate of  $\text{Ca}^{2+}$  through the plasma membrane (Mauger et al., 1984).



**Figure 2.** Initial  $^{45}\text{Ca}$  uptake by wild type (*a* and *c*), *pwa* (*b* and *d*), and *tam8* (*e*) cells in the presence (*black squares*) and absence (*open squares*) of AED at 26°C (*a*, *b*, and *e*) or 0°C (*c* and *d*). For stimulated cells, AED was added together with  $^{45}\text{Ca}$ . Each point is the mean of two measures. At 26°C, the initial rate of  $^{45}\text{Ca}$  uptake (as measured at 5 s in *a* and *b* and at 4 s in *e*) is multiplied by a factor 9–11 in the three strains when AED is present, as observed in 7, 4, and 1 independent experiments for the wild type, *pwa*, and *tam8*, respectively. No stimulation of  $^{45}\text{Ca}$  uptake is observed when AED is applied at 0°C.

Therefore, the initial rate of  $^{45}\text{Ca}$  uptake was measured in the presence or absence of the triggering agent and the  $\text{Ca}^{2+}$  influx calculated from the linear part of the  $^{45}\text{Ca}$  uptake curve (2–5 s, according to the experiment). As shown on Fig. 2, *a* and *b*, the initial rate of  $^{45}\text{Ca}$  uptake measured at 26°C for nonstimulated cells is  $\sim 1$  pmol/s/1,000 cells and 10-fold higher for stimulated cells. When the same experiment is carried out at 0°C (Fig. 2, *c* and *d*), a temperature that inhibits exocytosis, the initial rate of  $^{45}\text{Ca}$  uptake from AED-stimulated cells is not increased as compared to that from nonstimulated ones. AED-triggered exocytosis and  $^{45}\text{Ca}$  uptake are therefore correlated and thermo-dependent.

To check whether this uptake could reflect an artefactual binding of  $^{45}\text{Ca}$  on released trichocyst matrices or diffusion from the external medium into the trichocyst ghost vesicles during the transient opening which accompanies exocytosis, we performed  $^{45}\text{Ca}$  uptake experiments on the mutant *tam8*, whose trichocysts are not attached to the plasma membrane and cannot be released, owing to a mutation that has been localized to the trichocyst compartment (Aufderheide, 1978).

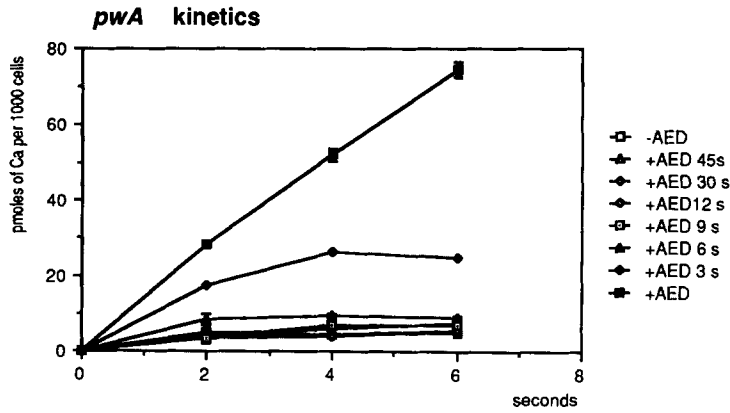
Fig. 2 *e* shows that, although no exocytosis could occur in *tam8*, AED was able to induce the same 10-fold increase in  $^{45}\text{Ca}$  uptake as in wild type cells. This indicates (*a*) that the observed  $^{45}\text{Ca}$  uptake indeed represents a  $\text{Ca}^{2+}$  influx, not binding to excreted trichocysts; and (*b*) that this influx, still present in the mutant, is not mediated by transient contacts between the external medium and the interior of the vesicles during membrane fusion.

To measure the duration of the AED-induced  $^{45}\text{Ca}$  influx, we added  $^{45}\text{Ca}$  to the cells at different times after the stimulation and measured the initial rate of calcium uptake in wild type and *pwa* cells. As illustrated in Fig. 3 for *pwa*, the initial rate of  $^{45}\text{Ca}$  uptake quickly decreases (it is reduced by half in 2–3 s) and returns to the basal rate within  $\sim 10$  s. The observed  $\text{Ca}^{2+}$  influx induced by AED is therefore rapidly regulated.

#### *Ca<sup>2+</sup> Influx in nd Mutants*

When exocytosis is triggered, wild type cells as well as *pwa*

a



b

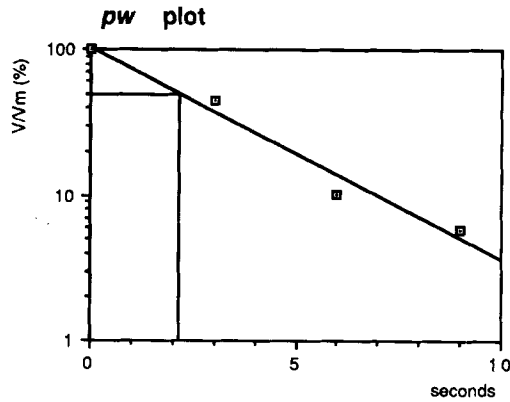


Figure 3. Initial  $^{45}\text{Ca}$  uptake at various times after AED stimulation in pwa cells (a). Initial  $^{45}\text{Ca}$  uptake kinetics at 0, 3, 6, 9, 12, 30, and 45 s after AED stimulation. The cells are filtered 2, 4, or 6 s after  $^{45}\text{Ca}$  addition. (b) Initial rates of  $^{45}\text{Ca}$  incorporation from Fig. 3 a (measured at 2 s) expressed as a percent of the maximal rate (AED added without delay) and plotted against the delay between stimulation and  $^{45}\text{Ca}$  addition. In this experiment, the AED-induced influx was reduced by half in  $\sim 2$  s.

cells (despite their lack of voltage-operated channels) show backward swimming from a half to a few seconds in response to transient elevation of  $[\text{Ca}^{2+}]_i$  above  $6 \times 10^{-7}$  M (see Materials and Methods). Mutants representative of the 12 known *nd* genes all displayed backward movements upon AED stimulation (Bonnemain, H., and J. Cohen, unpublished observation) as also observed for three of these *nd* mutants by Plattner et al. (1985) and by Matt et al. (1980) with other secretagogues. However, all these mutants possess normal voltage-operated  $\text{Ca}^{2+}$  channels in their cilia since they are  $\text{PW}^+$ , and the behavior observed after the stimulation of exocytosis could result from the opening of these channels. To detect alterations due to *nd* mutations without interference from voltage-operated  $\text{Ca}^{2+}$  channels, we constructed pwa-*nd* double mutants (see Materials and Methods) and observed their swimming reactions and  $^{45}\text{Ca}$  uptake upon triggering of exocytosis by AED.

Among the six pwa-*nd* double mutants constructed, three (pwa-*nd3*<sup>c</sup>, pwa-*nd6*, pwa-*nd9*<sup>a</sup>) displayed normal backward swimming upon contact with AED. pwa-*nd7* and pwa-*nd169* show significantly shorter backward swimming upon stimulation, while pwa-*nd12* never showed any backward swimming when cells were grown at 35°C, the nonpermissive temperature for *nd12*. The observed correlation between exocytotic capacity and backward swimming in the same strain (pwa-*nd12*), depending on the culture conditions, strongly suggests the idea that both phenomena are related to the same event.

In  $^{45}\text{Ca}$  uptake experiments, five pwa-*nd* mutants (pwa-*nd3*<sup>c</sup>, -*nd6*, -*nd7*, -*nd9*<sup>a</sup>, -*nd169*) presented a normal  $\text{Ca}^{2+}$  influx when stimulated with AED (shown in Fig. 4 for pwa-*nd7* and pwa-*nd9*<sup>a</sup>). Only one of them, pwa-*nd12*, when grown at the nonpermissive temperature (35°C), displayed no AED-inducible  $^{45}\text{Ca}$  uptake (Fig. 5 b), although pwa (Fig. 5 a), and wild type (not shown) cells grown at the same temperature presented the same AED-induced uptake, as observed at lower temperatures.  $^{45}\text{Ca}$  uptake experiments were also performed on the corresponding single *nd* mutants (data not shown) with the same result as obtained with the double mutants. The gene *nd12*, therefore, seems a good candidate to encode a protein involved in the regulation of membrane permeability to  $\text{Ca}^{2+}$ . Here, too, the correlation between exocytotic capacity and the increase in  $^{45}\text{Ca}$  uptake according to the temperature of the cell culture supports the idea that the uptake triggered by AED is physiological and directly related to exocytosis.

## Discussion

In *Paramecium* trichocyst exocytosis can be equated to membrane fusion and therefore provides a model system for studying this ubiquitous but obscure phenomenon. An important unresolved question is whether  $\text{Ca}^{2+}$  is necessary or not for membrane fusion in vivo. Trichocyst exocytosis is accompanied by ciliary reversal detected as backward swimming, a behavior known to be directly dependent on a rise

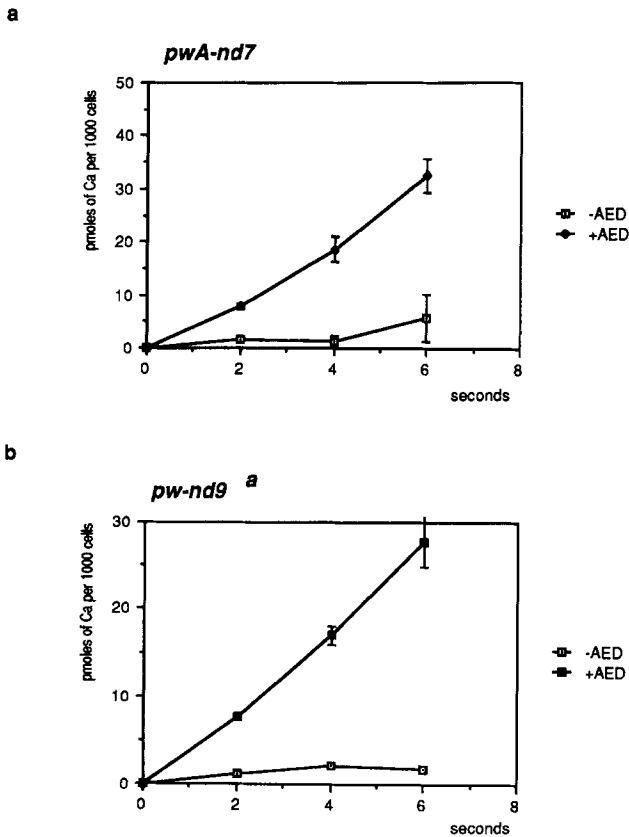


Figure 4. Initial  $^{45}\text{Ca}$  uptake by pwA-nd7 (a) and pwA-nd9a (b) cells. See the legend of Fig. 2.

of  $[\text{Ca}^{2+}]$ , to a concentration superior to  $6 \times 10^{-7}$  M (see Materials and Methods). This  $\text{Ca}^{2+}$  can a priori arise either from internal stores or from the extracellular medium or from both by a "calcium-induced calcium release" mechanism (Berridge and Galione, 1988; Lipscombe et al., 1988). We have measured the  $\text{Ca}^{2+}$  movements associated with exocytosis in *Paramecium* and we have taken advantage of the genetic properties of the organism by analyzing mutations affecting on the one hand exocytosis (nd) and on the other hand ciliary voltage-operated  $\text{Ca}^{2+}$  channels (pw). The properties of the mutants are summarized in Table I.

Our results demonstrate that (a)  $3 \times 10^{-7}$  M external  $\text{Ca}^{2+}$  is required for induction of trichocyst release by the triggering agent AED; and (b) a massive  $\text{Ca}^{2+}$  influx is induced by AED. Most interestingly, one of the exocytosis mutations studied, nd12, specifically affects this influx.

#### Trichocyst Exocytosis Is Accompanied by a Transient $\text{Ca}^{2+}$ Influx

In this study, carried out on wild type, pwA, and a series of nd mutants unable to undergo exocytosis (as well as the corresponding pwA-nd double mutants), we observed that (a) steady-state measurements display a biphasic incorporation curve upon AED stimulation where significant  $\text{Ca}^{2+}$  uptake lasting almost 1 min is followed by  $^{45}\text{Ca}$  extrusion; (b) the initial rate of  $^{45}\text{Ca}$  uptake is increased 10-fold when AED is added; and (c) the AED-induced  $^{45}\text{Ca}$  uptake has a half-life of 2–3 s. As explained in the Results, initial rates of  $^{45}\text{Ca}$  uptake can be equated to  $\text{Ca}^{2+}$  influx, without interference

from efflux or internal redistributions, provided that the kinetics are performed over times sufficiently short compared to the time constant of  $\text{Ca}^{2+}$  exchanges (Mauger et al., 1984), which in our experiments stands between 5 and 10 min (see two examples in Fig. 1). The results presented here, therefore, demonstrate that AED induces a marked  $\text{Ca}^{2+}$  influx concomitant with trichocyst exocytosis. However, the molecular support of this influx (channel, exchanger . . .) cannot be deduced from our study and awaits an electrophysiological approach.

As external free  $[\text{Ca}^{2+}]$  is needed and as  $\text{Ca}^{2+}$  enters the cell upon AED induction, and although the time range of our influx measurements is much longer than exocytosis itself (a few milliseconds), a cause-effect relationship can be proposed between the  $\text{Ca}^{2+}$  influx and exocytotic membrane fusion. Indeed, on the one hand, deprivation of external  $\text{Ca}^{2+}$  instantaneously inhibits exocytosis as EGTA present in the AED sample prevents trichocyst release and, on the other hand, in experiments where cells are preequilibrated in Ca/EGTA buffers, a minimum of  $3 \times 10^{-7}$  M free  $[\text{Ca}^{2+}]$  is necessary for AED-triggered exocytosis. We favor the hypothesis that the dependence of exocytosis upon external  $\text{Ca}^{2+}$  reflects its dependence upon the  $\text{Ca}^{2+}$  influx and, therefore, that this influx takes place before membrane fusion. This hypothesis however does not exclude the possibility that  $\text{Ca}^{2+}$  liberated from internal stores acts cooperatively with entering  $\text{Ca}^{2+}$  to promote membrane fusion.

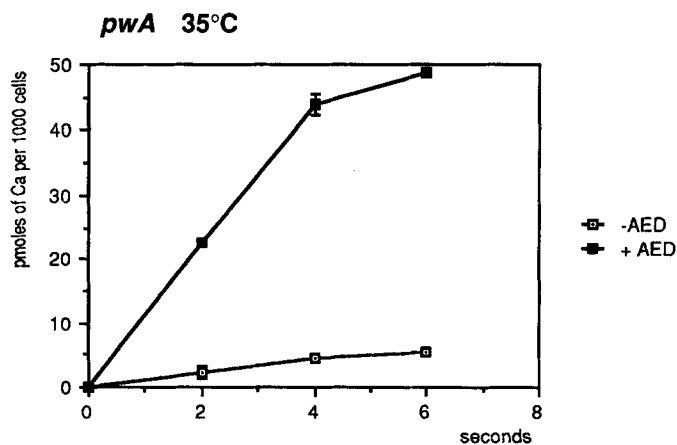
The time necessary for membrane permeability to  $\text{Ca}^{2+}$  to return to its basal level after AED stimulation is much longer than trichocyst release itself. This could reflect either the inertia of the cell upon physiological disturbance or an incomplete synchrony between individual events. This could also be due to events following exocytosis such as membrane resealing, vesiculation of the trichocyst ghost membrane, and recycling of these membranes (Hausmann and Allen, 1976; Allen and Fok, 1980). As reported and discussed by Foskett et al. (1989) and Cheek (1989) for mammalian secretory cells, what seems important for triggering membrane fusion is an initial local  $[\text{Ca}^{2+}]$  increase rather than a global concentration increase in the cell, which spans longer times.

Interestingly, the time course of the AED-induced  $\text{Ca}^{2+}$  influx approximately fits that of the transient dephosphorylation (Ziesenis and Plattner, 1985) of a 65-kD phosphoprotein first described by Gilligan and Satir (1982). This situation strongly resembles what happens with voltage-operated  $\text{Ca}^{2+}$  channels of *Paramecium* where the transient dephosphorylation of a phosphoprotein is necessary for regulation of the channel (Klumpp et al., 1990). In our system, the 65-kD phosphoprotein dephosphorylation may turn out to be a mechanism of regulation of the change in membrane permeability. Alternatively, this dephosphorylation could be provoked by the  $\text{Ca}^{2+}$  influx, as proposed by Momayesi et al. (1987).

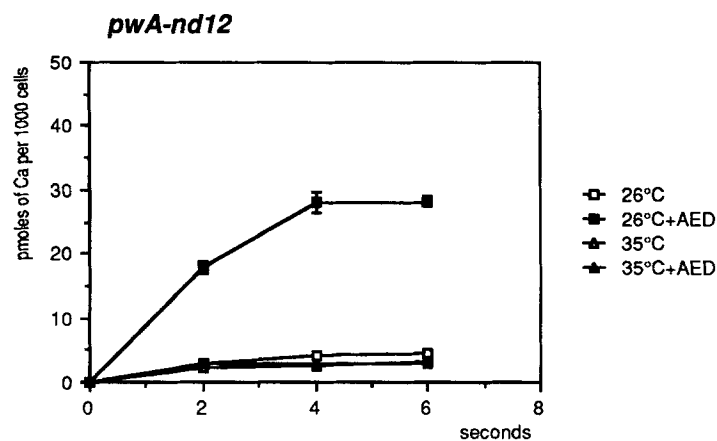
#### The $\text{Ca}^{2+}$ Influx Is Abolished in an nd Mutant

In contrast to all the other nd mutants tested, the mutant nd12 is deficient in both the  $\text{Ca}^{2+}$  uptake and the intracellular  $[\text{Ca}^{2+}]$  elevation associated with AED stimulation when cultured at the temperature ( $35^\circ\text{C}$ ) which prevents exocytosis, but not at lower temperatures where exocytosis is possible (Table I). Although the lipid composition of the membrane

a



b



**Figure 5.** Initial  $^{45}\text{Ca}$  uptake by pwA cells grown at 35°C (a) and by pwA-nd12 (b) grown at 26°C, which is a permissive temperature, and at 35°C which is the nonpermissive temperature. Note that AED provokes a dramatic increase of initial rate of  $^{45}\text{Ca}$  uptake only at 26°C and not at 35°C, although this is possible at that temperature in the absence of the nd12 mutation (a).

could be different at the nonpermissive temperature, the lack of  $\text{Ca}^{2+}$  uptake at 35°C in the mutant is highly significant since control wild type or pwA cells grown at that temperature do take up  $\text{Ca}^{2+}$  upon AED stimulation at the same rate as at 26°C or even slightly faster. The fact that nd12 is the only mutation, among the six nd mutants tested, which affects AED-induced  $\text{Ca}^{2+}$  movements strongly, suggests that the *ND12*<sup>+</sup> gene specifically encodes a factor involved in the regulation of membrane permeability to  $\text{Ca}^{2+}$  (e.g., opening of a channel) or to an event induced by AED which lies upstream of  $\text{Ca}^{2+}$  influx in the stimulation sequence

(e.g., reception of AED, signal transduction, generation of a second messenger). To our knowledge, it is the first time that such a mutation, affecting  $\text{Ca}^{2+}$  influx involved in membrane fusion in exocytosis, has been described.

#### *Exocytosis-specific $\text{Ca}^{2+}$ Channel?*

The massive  $\text{Ca}^{2+}$  influx associated with AED-induced exocytosis could occur through the opening of a novel specific  $\text{Ca}^{2+}$  channel. As mentioned in the Introduction, secretion strongly depends on  $\text{Ca}^{2+}$  and channels exist that are specialized in  $\text{Ca}^{2+}$  entry for particular physiological responses.

**Table I. Properties of the Different Mutants Studied in this Work**

| Strains   | Ciliary reversal in depolarizing medium | Trichocyst release | Ciliary reversal upon contact with AED | AED-stimulated $\text{Ca}^{2+}$ influx |
|---|---|--------------------|--|--|
| Wild type   | yes                                     | yes                | yes                                    | yes                                    |
| pwA   | no                                      | yes                | yes                                    | yes                                    |
| nd3 <sup>c</sup> , nd6, nd7, nd9 <sup>a</sup> , nd169                     | yes                                     | no                 | yes                                    | yes                                    |
| pwA-nd3 <sup>c</sup> , pwA-nd6, pwA-nd7, pwA-nd9 <sup>a</sup> , pwA-nd169 | no                                      | no                 | yes                                    | yes                                    |
| nd12 26°C   | yes                                     | yes                | yes                                    | yes                                    |
| 36°C  | yes                                     | no                 | yes                                    | no                                     |
| pw-nd12 26°C  | no                                      | yes                | yes                                    | yes                                    |
| 36°C  | no                                      | no                 | no                                     | no                                     |

Different types of channels have been reported that respond to different stimuli, voltage-operated channels, receptor-operated channels, and second messenger-operated channels (Meldolesi and Pozzan, 1987), as well as mechano-sensitive ion channels (Kung et al., 1990). It is unlikely that in *Paramecium* exocytosis depends on novel voltage-operated channels, since local application of AED triggers trichocyst discharge only in the restricted area of contact (Plattner et al., 1984) without propagation resembling the action potentials observed in ciliary reversal (Satow and Kung, 1980). Whether this putative  $\text{Ca}^{2+}$  channel is directly activated by AED or via a second messenger remains to be established. The existence of a  $\text{Ca}^{2+}$  channel specific for trichocyst exocytosis has already been proposed, mainly because inhibition of exocytosis has been obtained using the  $\text{Ca}^{2+}$  blockers verapamil and nifedipine (Maleki et al., 1987). However, these results were not confirmed for nifedipine (see Satir et al., 1988) and were in contradiction with those of Matt et al. (1978) who found stimulation (not inhibition) of exocytosis with D600, an analog of verapamil. The pharmacology of these putative channels is an open problem.

In addition, from the study of different nd mutants, we do not observe any correlation between the  $\text{Ca}^{2+}$  influx and the presence of an organized array of intramembranous particles, the rosette (see Materials and Methods). Indeed, nd12 possesses a normal rosette at nonpermissive temperature (Pouphile et al., 1986) but lacks the  $\text{Ca}^{2+}$  influx, whereas the 5 other nd mutants tested as well as the tam8 mutant lack the rosette but display normal AED-induced  $\text{Ca}^{2+}$  influx. Therefore, the hypothesis that the assembly of the rosette initiates a  $\text{Ca}^{2+}$  channel activity, as initially proposed by Satir and Oberg (1978), is most unlikely.

To identify the gene products responsible for  $\text{Ca}^{2+}$  entry and those involved in membrane fusion, and to investigate the nature of possible second messenger(s) and of their target(s), as well as of  $\text{Ca}^{2+}$  target(s), in vitro systems have to be worked out for direct access to the exocytotic site, either using permeabilized cells or lyzed cells which retain their exocytotic performance (Vilmart-Seuwen et al., 1986) or by reconstituting models from cellular fractions such as cortices (Keryer et al., 1990) and trichocysts isolated with their membranes (Lima et al., 1989).

We are indebted to Brigitte Berthon and Michel Claret (Université Paris XI Orsay) for their help in developing  $^{45}\text{Ca}$  uptake measurements and in interpreting flux results, and to Janine Beisson (Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette) for precious advice throughout this work. We thank Janine Beisson, Michel Claret, Peter Tatham (University College, London), and Linda Sperling (Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette) for critical reading of the manuscript.

A fellowship from the Ministère de la Recherche et de la Technologie to D. Kerboeuf and financial supports from the Centre National de la Recherche Scientifique, the Ligue Nationale Française contre le Cancer, and the Fondation pour la Recherche Médicale are gratefully acknowledged.

Received for publication 15 June 1990 and in revised form 6 August 1990.

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