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Aspects of Signal Transduction in Stimulus Exocytosis-Coupling in *Paramecium*

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This paper deals with the detailed mechanisms of signal transduction that lead to exocytosis during regulative secretion induced by specific secretagogues in a eukaryotic cell, *Paramecium tetraurelia*. There are at least three cellular compartments involved in the process: I) the plasma membrane, which contains secretagogue receptors and other transmembrane proteins, II) the cytoplasm, particularly in the region between the cell and secretory vesicle membranes, where molecules may influence interactions of the membranes, and III) the secretory vesicle itself.

The ciliated protozoan *Paramecium tetraurelia* is very well suited for the study of signal transduction events associated with exocytosis because this eukaryotic cell contains thousands of docked secretory vesicles (trichocysts) below the cell membrane which can be induced to release synchronously when triggered with secretagogue. This ensures a high signal-to-noise ratio for events associated with this process. Upon release the trichocyst membrane fuses with the cell membrane and the trichocyst content undergoes a Ca^{2+}-dependent irreversible expansion. Secretory mutants are available which are blocked at different points in the signal transduction pathway.

Aspects of the three components mentioned above that will be discussed here include a) the properties of the vesicle content, its pH, and its membrane; b) the role of phosphorylation/dephosphorylation of a cytosolic 63-kilodalton (kDa)M_r protein in membrane fusion; and c) how influx of extracellular Ca^{2+} required for exocytosis may take place via exocytic Ca^{2+} channels which may be associated with specific membrane microdomains (fusion rosettes).

Key words: secretion, cell membrane, calcium channels, membrane fusion, phosphoproteins

We are investigating the detailed mechanisms of signal transduction that lead to exocytosis during regulative secretion [1] induced by specific secretagogues in eukaryotic cells. The subject will be discussed with specific reference to the ciliated protozoan *Paramecium tetraurelia*. There are at least three cellular compartments involved in the process: 1) the plasma membrane, which contains receptors to recognize the secretagogue and a variety of transmembrane proteins whose actions influence the organization of the underlying cytoplasm; 2) the cytoplasm, particularly

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in the region between the cell and secretory vesicle membrane, where soluble messengers, enzymes, and other molecules may influence interaction of the membranes; and 3) the secretory vesicle itself, which must be addressed to the appropriate fusion-competent site below the plasma membrane. The properties of the vesicle content and its membrane are also crucial to the specification of the interactions that finally accompany product release.

For many years, the general paradigm for exocytosis was derived from the classical studies of the neuromuscular junction [2], that an extracellular stimulus induces membrane depolarization, opening of voltage-sensitive calcium channels, and an increase in cytoplasmic free Ca^{2+} concentration. The situation now seems considerably more complicated, since several different classes of Ca^{2+} channels are now known, messengers other than Ca^{2+} are well established, and a source of the rise in cytoplasmic Ca^{2+} may not always be extracellular. In addition, even where, as in *Paramecium*, it can be shown that Ca^{2+} plays a key role in exocytosis, the mechanisms by which an increase in cytoplasmic Ca^{2+} concentration produces fusion of the partner membranes at the docking site of secretory cells and actual release of product has been under investigation for many years and still is not known in detail.

One reason for the difficulty in sorting out exocytosis lies in the complexity of organization of the numerous Ca^{2+}-sensitive processes which occur in the cortex of the cell [3]. We suggest that the organization of membrane microdomains such as rosettes, cilia necklaces, gap, and tight junctions [4] is one way to permit differences in pCa (where pCa = $-\log [Ca^{2+}]$) to develop between compartments of the cell cortex. When cytoplasmic pCa decreases, it occurs site-specifically, triggering only what is being stimulated (for example, an exocytic event but not backwards swimming or change in cell shape). This spacial resolution of Ca^{2+} would also affect the location of Ca^{2+}-binding proteins such as calmodulin (CaM), which would be involved with the rapid containment of the Ca^{2+} signal and activation of specific enzymes. These enzymes could be associated with the membranes themselves or with the cytoskeleton in the vicinity of the fusion site. For example, Ca^{2+} could activate specific lipases [5], methyltransferases [6,7], protein kinases, phorphorylases, etc, to bring about local changes in phospholipid compositions or concentrations, or to bring about specific covalent modifications, e.g., phosphorylation or dephosphorylation, of structural proteins. Ca^{2+} often induces such effects via Ca^{2+}-CaM complex formation. One example of this is the smooth muscle stimulus-contraction cascade where Ca^{2+}-CaM activates myosin light chain kinase, and phosphorylation of myosin increases its interaction with actin [8].

Phosphorylation of the Ca^{2+} channel or pump proteins self-regulates the cytoplasmic pCa and consequently the intracellular signals [9,10]. Most of the studies reporting modulation of Ca^{2+} channel activity have involved cAMP-dependent phosphorylation [9], but modulation of Ca^{2+} channels via protein kinase C has recently been reported [11]. This is especially interesting in view of new ideas which have developed within the last few years involving the role of a relatively minor group of phospholipids, the polyphosphoinositides. These may play a role in signal transduction for a wide variety of transmitters, hormones, and growth factors. This system, when activated by ligand-binding to receptors, activates polyphosphoinositide phosphodiesterase (PPI-E) via guanine nucleotide binding proteins (Gp). Inositol phosphates are released which can mobilize Ca^{2+} from internal stores, probably endoplasmic reticulum cisternae. Diacylglyerol (DAG), an activator of protein kinase C, is also generated [12–14].

GENERAL BACKGROUND OF THE SYSTEM

The system in which we study the details of signal transduction during exocytosis is the single-cell ciliate *Paramecium tetraurelia*, because of the speed and synchrony with which exocytosis can be induced, the unique structural features which enhance the possibility of characterizing the critical components of each compartment in this system, and the availability of signal transduction mutants that fail to release properly (Table I).

The *Paramecium tetraurelia* cortex consists of rows of cortical units, which measure about 1–1.5 μm^2, each containing a potential site for exocytosis (Fig. 1.) The cell is ca 150 μm long and 50 μm in diameter, and several thousand secretory events per cell can be induced synchronously within milliseconds after application of trinitrophenol (TNP), the routine stimulus, which means that the signal-to-noise ratio of any event associated with exocytosis is very high.

The *Paramecium* secretory vesicle of interest, the trichocyst, is a membrane-bounded organelle which contains a condensed protein matrix. We shall use the designation "trichocyst" solely for the cytoplasmic, membrane-bounded secretory vesicle (Fig. 2, arrowheads), and "trichocyst matrix" (tmx) for the secreted content (Fig. 2, arrows). Upon stimulation with a secretagogue, when fusion of plasma and trichocyst membranes occurs, the tmx expands and leaves the cell as a paracrystalline structure about eight times its original length. Secretion is easily detected because the expanded tmx is visible in the light microscope as a 20–40-μm-long needle. Thus secretory events in individual cells or parts of cells can be individually quantitated, and population behavior can be studied by direct counts or by spectroscopic methods. The tmx undergoes a calcium-induced reordering of its paracrystalline structure during release, and we have defined three stages in this expansion process. The stage I, or fully condensed, tmx, is the 4-μm-long, membrane-bounded form existing prior to stimulation. Stage II, the partially expanded tmx, is defined as an intermediate stage in the transition, preceding stage III, the fully expanded, extruded form, which is the 20–40-μm-long needlelike structure [15].

In *Paramecium*, prior to the actual fusion event that signals exocytosis, the secretory vesicles (trichocysts) dock at specific sites below the cell membrane. The fusion rosette, a structurally well-characterized plasma membrane microdomain, whose freeze-fracture signature is an intramembrane particle (IMP) array, then assembles in the cell membrane above the docked trichocyst (Fig. 1, arrows). When a secretory stimulus is presented to the cell, membrane fusion and exocytosis occur at the site of the rosette. Although presumably a multiplicity of cytoplasmic factors and transmembrane signals are involved in the steps leading to exocytosis, the rosettes stand in a key position along the pathway. Successful membrane fusion and exocytosis have never been observed in the absence of the rosette in either *Paramecium* or *Tetrahymena* [16–20].

TABLE I. Three Classes of Secretory Mutants

I.	*Signal transduction mutants:* transport and dock trichocysts below the cell membrane but do not release them upon stimulation (nd9, nd6) [18,66]
II.	*Transport mutants:* deficient in vesicle transport to the docking site (tam-8) [67]
III.	*Synthesis mutants:* totally devoid of trichocyst (tl) [68]

Fig. 1. This micrograph represents an unfixed, quick-frozen unidirectional shadowed sample of a *Paramecium* prepared in collaboration with Dr. John Heuser. The freeze-fracture micrograph shows the PF of the cell membrane of *Paramecium*. Many of the exocytic membrane microdomains can be seen showing the outer ring of particles (arrowheads) with the fusion rosette in the center (arrows). Cross-fractured cilia (Ci) delineate the longitudinal meridians. ×75,000.

Fig. 2. This micrograph represents an unfixed, quick-frozen, deep-etched specimen. Two trichocysts are seen docked below the cell membrane (arrowheads). The trichocysts have been cross-fractured and the semi-crystalline content is clearly visible (arrows). A cilium (Ci) is located in between the two trichocysts. Preparation made in collaboration with Dr. John Heuser. ×60,000.

CALCIUM EFFECTS IN *PARAMECIUM*

In *Paramecium* three Ca^{2+}-mediated effects have been demonstrated: 1) a complex effect on exocytosis [3, 15, 21–23], 2) an effect on ciliary beat [24–26], and 3) an effect on cell shape [26]. In addition, there are other possible Ca^{2+}-sensitive processes occurring in the *Paramecium* cell cortex, including endocytosis. Moreover, the cell is well studied electrophysiologically, the properties of voltage-sensitive Ca^{2+} channels are well understood, and mutants which do not possess this channel (pawn) are known [27].

The critical range of pCa is known for several of these processes in *Paramecium*. Normally cytoplasmic Ca^{2+} concentration in *Paramecium* is 10^{-7} M (pCa 7) or lower. Extracellular Ca^{2+} is normally in the millimolar range. Increases in cytoplasmic Ca^{2+} from extracellular sources depend upon this gradient across the cell membrane, and a rise in the cytoplasmic free Ca^{2+} towards pCa 5 initiates response. These concentrations are comparable to the physiological range of effects in other cells. In living *Paramecium* the cilia provide an internal in vivo monitor of pCa, because there has been shown to be a direct correspondence between pCa and swimming speed in this cells [3, 24].

CALCIUM EVENTS ASSOCIATED WITH THE SECRETORY VESICLE

The role of Ca^{2+} in exocytosis is complex, but as discussed earlier, a likely candidate for mediating its action is the calcium-dependent regulatory protein, calmodulin (CaM). We found that CaM is present in *Paramecium* in both cytoplasmic fractions [28,29] and localized to specific cellular structures [30]. Its involvement in the process of tmx secretion has been under investigation in this laboratory and others [15, 31–35].

Initial experiments indicated that incubation of *Paramecium* in 40 μM trifluoperazine (TFP), a drug known to inhibit CaM-regulated enzymes [36], inhibited TNP-induced tmx release [29]. Using the three defined stages of tmx expansion, we [15] tested the effects of the CaM antagonists TFP and W-7 (naphtalenesulfonamide), and W-5 (the less active dechlorinated analog of W-7, which serves as a control) on trichocyst matrix expansion in vivo. The first two reversibly inhibited secretion, whereas W-5 had no effect.

Ultrastructural examination of these cells revealed that a specific calcium-dependent step in the release process, expansion of the tmx from stage I to stage II, was inhibited. From these results we suggested that matrix expansion was blocked in vivo because CaM antagonists limited the access of Ca^{2+} to the matrix [15]. However, a possible mechanism for CaM antagonist action that had not been ruled out was a direct interaction of these agents with the tmx proteins themselves. In approaching this problem, we used an in vitro preparation of isolated, membrane-free condensed tmx's in combination with a rapid, quantitative, spectrophotometric assay for expansion based on the turbidity change that accompanies the stage I to stage III transition [22]. Using a series of defined buffers at pCa's, we showed that there was little change in OD_{320} with decreasing pCa to pCa 6.0. Below pCa 6.0, a rapid drop in OD_{320} occurred, which was essentially complete by pCa 5.5. Increasing Ca^{2+} concentrations further had little effect. Light microscope examination of samples of different pCa's indicated that the drop in OD_{320} corresponded to tmx expansion. CaM

antagonists did not affect in vitro tmx expansion; therefore, tmx proteins could be eliminated as a site of action for these agents in vivo.

In addition, a pronounced effect of pH on the calcium-induced in vitro expansion was found. At low pH (lower than 6.0) tmx expansion required higher Ca^{2+} concentration, whereas at pH greater than 7.0, expansion was facilitated (i.e., it occurred at a lower Ca^{2+} concentration). From these results, it was suggested that, in vivo, intratrichocyst pH is acidic, and that a primary function of low intratrichocyst pH may be to maintain trichocyst proteins in their condensed storage form [22].

MODEL HYPOTHESIZED FOR VESICLE CHANGES

From the in vivo and in vitro results, we have devised a hypothesis for some of the regulatory events, at the level of the secretory vesicle, which must take place in coordination with membrane fusion in *Paramecium* for a successful release event to take place [22].

In the unstimulated condition (a), cytoplasmic free Ca^{2+} (Ca_i^{2+}) is lower than 10^{-7} M, CaM is largely Ca^{2+}-free, and the tmx is condensed (stage I). The interior of the trichocyst vesicle may be maintained at a low pH by a proton pump in the trichocyst membrane. Within the acidic secretory granule, the interaction of H^+ with the trichocyst proteins presumably keeps them in a condensed form, thereby preventing matrix expansion. After stimulation (b), Ca_i^{2+} rises above 10^{-6} M and cytoplasmic Ca^{2+}-CaM complexes are formed. These complexes are thought to be the primary target for CaM antagonists, although other cellular targets cannot be ruled out.

Ca^{2+}-CaM complexes are presumed to act at the trichocyst membrane to control access of Ca^{2+} to the matrix. Whether CaM acts via direct binding to trichocyst membrane components, or via CaM-activated regulatory enzymes such as kinases or phosphatases, is not known (indicated by the black box). Transport of Ca^{2+} into the trichocyst may occur through a gate or channel in the membrane, or may be coupled to the outward movement of protons by an antiport. In this manner, stored energy in the form of a chemiosmotic gradient could be used to promote matrix expansion, while at the same time removing Ca^{2+} from the cytoplasm and thus terminating the signal for release. Ca^{2+} within the vesicle can then bind to sites on the tmx and lead to expansion. Membrane fusion must occur in a coordinated fashion with matrix expansion to allow release of secretory products to the extracellular space.

This hypothesis has been confirmed and expanded using living *Paramecium* (wild type [wt] and mutant cells) using 1) uptake of a basic amine, acridine orange (AO), in living *Paramecium* as an indicator of acidic compartments; 2) a series of secretory mutants for organelle identification; and 3) protonophores to show disappearance of the pH gradient across trichocyst membranes [37,38].

In earlier studies, Allen and Fok [39] found that AO accumulated in and specifically marked acidic digestive vacuoles (acidosomes) in *Paramecium*. Using this dye, we found that in addition to the acidosomes, AO is taken up and concentrated within vesicles which are observed as elongate, brightly stained bodies positioned below the cell membrane in a punctate pattern (Fig. 3). Three classes of secretory mutants have been studied. These are listed in Table I.

These mutants differ in the position of their trichocysts within the cytoplasm and have been used to test the hypothesis that the elongated bodies are trichocysts.

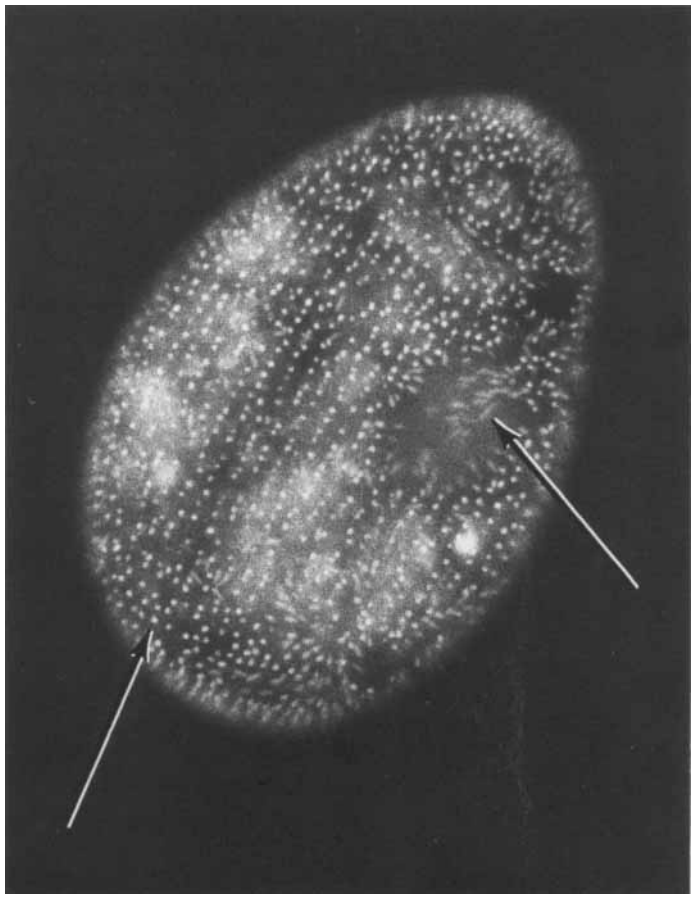

Fig. 3. This figure illustrates a live *Paramecium* which has accumulated the dye acridine orange within the trichocysts. The fluorescence of the trichocysts is seen either as rows of fluorescent dots (arrow) or longitudinally as small lines (arrow). This indicates that the trichocyst content has an acidic pH. ×730.

The positioning of these AO-stained bodies corresponded to the exact location of trichocysts in these mutant cell lines. In addition, the result also showed that the trichocysts of nd-6, nd-9, and tam-8 were not defective in their capacity to acidify the secretory organelles as judged by the AO staining technique. The AO staining of the tam-8 mutant indicates that acidification occurs during the maturation of the secretory vesicle prior to docking at the cortex of the cell. This suggests that a proton gradient may be involved in the condensation of the tmx proteins within the vesicle [38].

EFFECT OF PROTONOPHORES ON AO-STAINED TRICHOCYSTS IN LIVING *PARAMECIUM*

Addition of the protonophores carbonylcyanide, n-chlorophenylhydrazone (CCCP, 20 μM) or dinitrophenol (DNP, 1 mM) diminished the fluorescence staining in the secretory vesicles. In recovery experiments where the pronotophores were

successfully removed by washing, the AO fluorescence returned, indicating that the proton gradients of the trichocysts became reestablished. After treatment with CCCP, a challenge with the secretagogue TNP still induced exocytosis. In contrast, TNP-induced exocytosis was effectively blocked by even a short treatment (less than a minute) with DNP. The resemblance of the structure of DNP to TNP and the fact that the inhibitory effect of DNP is rapid (in contrast to dissipation of the proton gradient which takes many minutes), suggest that the inhibitory effect of DNP on exocytosis is probably due to molecular competition at a putative TNP receptor site rather than an effect on the trichocyst itself.

CCCP and DNP effect both proton gradient and ATP levels [40,41]. In order to separate the contribution of these effects on the secretory vesicle we used two drugs (sodium azide and chloroquine) in separate experiments. When wt cells are incubated in sodium azide (mM), which depletes ATP significantly in *Paramecium* [42] without a primary effect on proton gradients [43], the AO fluorescence within the trichocysts is diminished, but not as completely as by the protonophores. This indicates that the trichocyst relies on ATP for maintenance of its proton gradient. Upon stimulation with TNP in the presence of azide, normal exocytosis is seen. Chloroquine disrupts proton gradients [44]. The effect of chloroquine on ATP levels is not completely understood, but available evidence suggests that at 0.5 μM chloroquine, in the presence of glucose, ATP levels are diminished only slightly [45]. When AO-treated wild-type cells are incubated with 0.5 μM chloroquine, differential trichocyst staining is abolished. Upon TNP stimulation, normal exocytosis occurs even in the presence of chloroquine. These experiments imply that the proton gradient within the trichocyst is normally maintained by an ATP-dependent proton pump, presumably in the trichocyst membrane. Since abolition of the proton gradient across the trichocyst membrane does not inhibit stimulus-induced exocytosis, the gradient appears not to be necessary for the final stages of signal transduction in this process.

SEPARATION OF MEMBRANE FUSION AND tmx EXPANSION

Two major events in the secretory cascade are decondensation of secretory vesicle matrix and membrane fusion. The relationship between these events has not been clear, but we have now determined that decondensation can occur without concurrent membrane fusion.

We have succeeded in separating the process of tmx expansion from membrane fusion using the nd9 signal transduction mutant at the nonpermissive temperature (nd9-27) and the divalent cationophore A23187. When nd9 cells are stimulated with A23187 with sufficient Ca^{2+} in the medium, tmx expansion occurs, presumably because the ionophore permits Ca^{2+} to pass through the trichocyst membrane to reach the tmx, but membrane fusion does not take place. This event in mutant cells has been called "pseudoexocytosis" [46]. Since nd9-27 cells are missing the fusion rosettes [18], pseudoexocytosis suggests that the molecules represented by the rosette particles are necessary for membrane fusion [47]. Pseudoexocytosis is not unique to the mutant but can be seen in occasional single exocytic events in wt cells. In wild-type cells it is likely that pseudoexocytosis occurs because Ca^{2+} reaches the tmx before assembly of a complete rosette has taken place [47]. In wt cells, or nd9 cells at the permissive temperature (nd9-18), where docked trichocysts have complete fusion rosettes, addition of A23187-Ca^{2+} gives true exocytosis. It is possible, there-

fore, to raise the intracellular Ca^{2+} concentration in order to achieve both membrane fusion and tmx expansion when all components are normal.

Another way of separating tmx expansion and membrane fusion is via the use of a different secretagogue. Paranitrophenol (pNP), a structural analogue of trinitrophenol (TNP), has been studied for its ability to induce exocytosis in wt and nd9 cells. At the light microscope level, pNP apparently causes tmx release in wt and nd9-27 both in the presence and absence of extracellular Ca^{2+}. Therefore, the tmx release induced by pNP seems independent of both extracellular Ca^{2+} and the fusion rosette. However, when viewed in electron microscopy, the tmx's released by pNP are covered with membrane, indicating that tmx expansion has occurred without concomitant membrane fusion, i.e., that pseudoexocytosis has occurred. In contrast, TNP stimulates neither membrane fusion nor tmx expansion when extracellular Ca^{2+} and/or fusion rosettes are lacking. Thus TNP acts as a true secretagogue in that its effects are dependent on extracellular Ca^{2+} and the fusion rosettes, whereas pNP exerts its effect on the regulation of tmx expansion itself. These observations support the hypothesis that membrane fusion and expansion of the tmx are two distinct and independent events, which are tightly regulated so that both processes occur very nearly synchronously during true exocytosis.

The response in wt cells to pNP parallels that of ionophore in nd9 cell, the signal transduction mutant, in which even where external Ca^{2+} is not available, the ionophore induces tmx expansion. One possible interpretation of this result is that pNP acts in a manner similar to A23187, releasing Ca^{2+}, which enters the secretory vesicle to stimulate tmx expansion from intracellular source(s). TNP stimulation, which does not produce pseudoexocytosis, allows extracellular Ca^{2+} to enter the cytoplasm. Membrane fusion seems dependent on this event.

PLASMA MEMBRANE Ca^{2+} CHANNELS ARE ASSOCIATED WITH EXOCYTOSIS IN *PARAMECIUM*

It has long been known that replacing extracellular Ca^{2+} with Mg^{2+} blocks exocytosis in many secretory systems, including *Paramecium*. When extracellular Ca^{2+} is replaced by Mg^{2+} (20 mM), TNP-induced release of trichocysts is inhibited. Addition of Ca^{2+} (0.5 mM) to this solution restores the ability of the cells to release. The requirement for extracellular Ca^{2+} raises the question of whether specific Ca^{2+} channels are involved in the secretory response.

The organic Ca^{2+} channel blocker verapamil is known to block certain types of plasma membrane Ca^{2+} channels [48–51]. The secretory response to TNP of wt cells incubated in the presence of verapamil (30 mM) is inhibited within 10 min [52]. The kinetics of inhibition are dependent on drug concentration; i.e., the highest dose (75 μM) produces inhibition within 2 min, while 20 μM has no effect. The inhibitory effect of verapamil is in large measure reversible; i.e., washed cells regain secretory ability, whereas cells remaining in verapamil, although showing some autorecovery, remain mainly inhibited.

Verapamil does not block the ability of the tmx's to expand upon stimulation with pNP. In the presence of the highest levels of verapamil used, expansion of tmx's occurred at control levels with pNP. Therefore, verapamil specifically blocks membrane fusion, which requires influx of extracellular Ca^{2+}, tmx expansion does not necessarily depend on this influx. The data support the idea that Ca^{2+} necessary for

membrane fusion enters specifically via verapamil-sensitive, TNP-stimulatable exocytosis-associated Ca^{2+} channels in the cell membrane.

In the presence of nifedipine (50 μM), a dihydropyridine-type of Ca^{2+} channel blocker, no inhibition of TNP-stimulated exocytosis is observed [53], indicating that the Ca^{2+} channels associated with TNP-induced exocytosis are pharmacologically specific. The divalent cation cadmium was also tested [54]. Unlike verapamil, its Ca^{2+} antagonism arises from competition for Ca^{2+}-binding sites and an inability to permeate the Ca^{2+} channel [55–58]. TNP-stimulated exocytosis is blocked by 1–3 mM Cd^{2+} in the presence of 5 mM Ca^{2+} in our buffer system.

These exocytosis-specific Ca^{2+} channels differ from the well-known voltage-sensitive ciliary membrane Ca^{2+} channels. Pawn mutants are defective in ciliary Ca^{2+} channels [27]. In preliminary experiments we found that TNP-stimulated pawn mutants secrete normally [59].

MEMBRANE GRADIENT FOR EXOCYTOSIS

An unusual secretory response was observed for the first time in TNP-stimulated, verapamil treated cells: up to 50% of the cells were inhibited at the posterior end of the cell body but secreted from the anterior [52]. We termed this response "A^+P^-", corresponding to anterior end positive, posterior end negative for exocytosis. During the inhibitory response to verapamil, the A^+P^- category appears to be a transition stage before complete inhibition, where inhibition starts at the posterior end and proceeds towards the anterior end of the cell body. Likewise, recovery begins at the anterior end of the cell body and proceeds toward the posterior end. This A^+P^- category is also seen in verapamil-inhibited cells when tested with other secretagogues such as alcian blue and tannic acid, and therefore is not limited to TNP stimulation. These observations indicate that there is an anterior-posterior gradient of sensitivity to stimulation or blockage of exocytosis along the cortex of each individual *Paramecium* (Fig. 4). The structural and biochemical basis for this gradient is presently unknown [59].

A STIMULUS-SENSITIVE DEPHOSPHORYLATION OF A CYTOPLASMIC PHOSPHOPROTEIN

In 1982, Gilligan and Satir [60] demonstrated that when normal exocytosis is induced in wt cells, a rapid dephosphorylation of a M_r 63 kilodaltons (kDa) protein is detected (Fig. 5C). Preincubation of cells in high Mg^{2+} (no added Ca^{2+}) inhibits both exocytosis and dephosphorylation in response to TNP. Further, the temperature-sensitive (signal transduction) mutant nd9, when grown at 18°C (permissive temperature), where fusion rosettes at the secretory sites are normal, secretes normally and dephosphorylates the 63-kDa protein in response to TNP, but when grown at 27°C (nonpermissive temperature) where rosettes are not assembled, neither secretes nor dephosphorylates the 63-kDa M_r protein in response to the secretagogue.

This represents the first in vivo correlation between dephosphorylation of a specific 63-kDa phosphoprotein and exocytic activity in *Paramecium*. These results have been confirmed and extended by others to show that rephosphorylation normally takes place within 10 sec and that dephosphorylation of this protein does not occur in

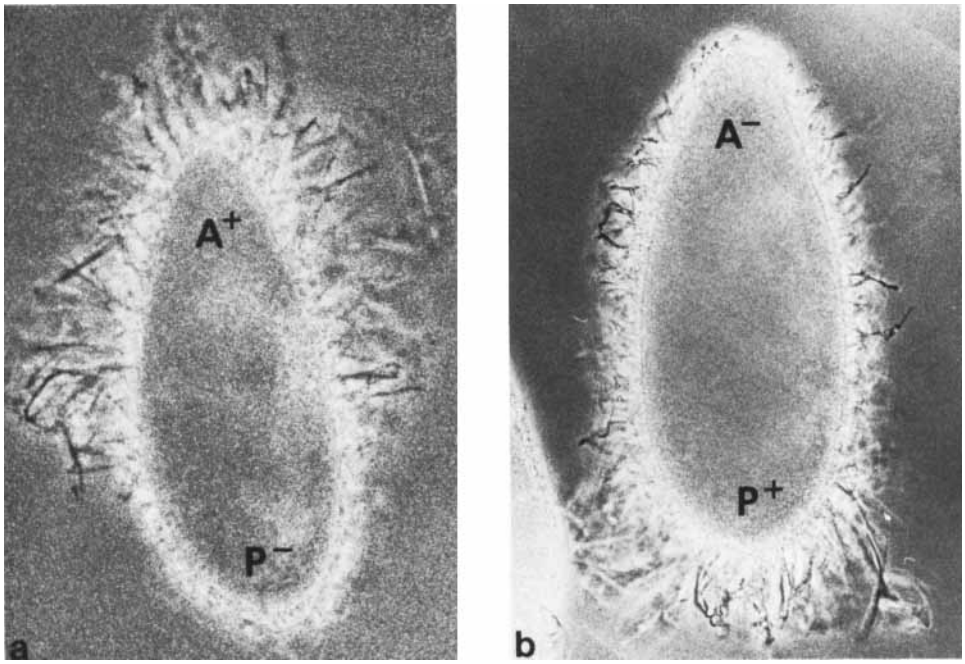

Fig. 4. Two light micrographs showing a verapamil- (**a**) and a Cd^{2+}- (**b**) treated cell after stimulation with TNP. Note the polarity of exocytic release. In the case of the verapamil-treated cell the release takes place only at the anterior end (A^+) of the cell. In contrast, the Cd^{2+}-treated cell releases only from the posterior end (P^+). ×576.

a series of other signal transduction mutants that show a defective exocytic membrane microdomain [61].

The phosphorylated form of the 63-kDa protein is found in the soluble, cytoplasmic fraction of homogenized cells (Fig. 5A). Therefore it is unlikely to be a component of the tmx, or an integral membrane protein (such as a rosette particle, secretagogue receptor, Ca^{2+} ion channel, or Ca^{2+}-ATPase).

^{32}P-labeled 63-kDa protein elutes from a gel filtration column with an apparent size of 60–65 kDa. The presence or absence of a disulfide reducing agent (such as beta-mercaptoethanol) has no effect on the M_r of the protein in SDS-PAGE. Therefore, it appears that the 63-kDa protein is a monomeric polypeptide in the native state.

The 63-kDa protein separates into multiple spots with pI's of 5.8–6.3 on two-dimensional (2D) PAGE, indicating that it is a slightly acidic protein which exists in multiple isolectric forms. Elution of ^{32}P-labeled 63-kDa polypeptide from preparative gels, followed by limited acid hydrolysis and 2D-thin-layer chromatography/electrophoresis, demonstrates predominantly radioactive phosphoserine residues [62].

The 63-kDa protein has been purified and used to generate a polyclonal antiserum from rabbits [62]. The affinity-purified antibody has been characterized by immunoprecipitation and by immunoblot analysis performed on ^{32}P-labeled cells and cell extracts. We found that the antibody precipitates the 63-kDa phosphoprotein from such extracts and binds almost exclusively to a band which exactly corresponds to the ^{32}P-labeled 63-Kda protein when cell extracts, separated, by SDS-PAGE, are trans-

ferred to nitrocellulose (Fig. 5B). Weak cross-reaction is also seen at 40-kDa band; it remains to be determined if this represents antibody binding to a different protein or to a fragment of the 63-kDa protein. With minor qualification, the affinity-purified antibody seems quite specific for the 63-kDa protein in *Paramecium* [62].

Immunoblot analysis of 2D-PAGE gels indicates that, in addition to the multiple spots of ^{32}P-labeled 63-kDa protein seen by autoradiography, the antibody also binds to an unlabeled (and presumably nonphosphorylated) form of 63-kDa protein. The ability to detect a nonphosphorylated form of 63-kDa protein, if it exists in the cell, is important since this is the form anticipated after exocytosis. Localization may be different for the phosphorylated and dephosphorylated protein, and therefore may provide a clue to its function. Phosphorylation is known to affect the assembly properties of proteins, particularly in membrane-cytomatrix interactions, for example, in nuclear membrane disassembly [63]. In addition, it has been shown with purified synaptic vesicles that synapsin 1, phosphoprotein of M_r 80 kDa, is bound to synaptic vesicles in its dephosphorylated form, but soluble when phosphorylated in a specific region [64]. The data in Figure 5 demonstrate that the 63-kDa protein can be detected in a 100,000g ("microsomal") pellet fraction by antibody binding. The pellet-associated 63-kDa protein is apparently not labeled with ^{32}P and therefore went undetected before antibody-binding analysis became available. This association of 63-kDa protein with some sedimentable cell component may represent another example of a phosphorylation-dependent translation of a protein.

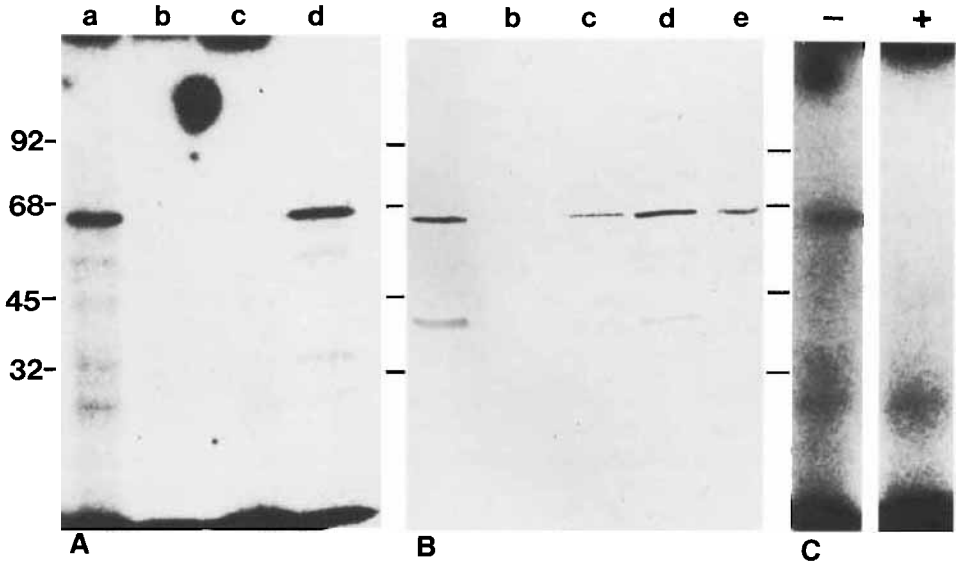

Fig. 5. Wild-type *Paramecium* cells were harvested in a Ca^{2+}-containing buffer and allowed to incorporate $^{32}P_i$ in vivo. The cells are then homogenized, centrifuged to produce a low-speed (1,500g) pellet, a high-speed (100,000g) pellet, and a soluble fraction. These fractions were then run on SDS-PAGE and transferred to nitrocellulose. **A:** ^{32}P autoradiogram of (**a**) homogenate, (**b**) low-speed pellet, (**c**) high-speed pellet, and (**d**) soluble fractions. **B:** Immunoblot showing binding of affinity-purified antibody against the 63-kDa protein to the same fractions. Lane e contains purified 63kDa protein as a standard. **C:** SDS-PAGE and ^{32}P-autoradiography of whole cells before (−) and after (+) stimulation with TNP.

A phosphorylation-dependent change in localization of the 63-kDa protein would suggest a regulatory, rather than structural, role for the protein. Since dephosphorylation of the 63-kDa protein apparently always accompanies normal exocytosis [60], defining this regulatory interaction could be quite critical for understanding the transduction cascade.

The availability of the antibody to the 63-kDa protein will now make it possible for us to localize the 63-kDa protein more completely. The availability of several mutants including the signal transduction mutant with a temperature-sensitive inability to dephosphorylate the 63-kDa protein should then permit better definition of the function of this protein.

Preliminary data [65] on indirect immunofluorescence localization of the 63-kDa protein in *Paramecium* wt cells have revealed a pattern reminiscent of the polygonal network characteristic of the *Paramecium* cortex. At the moment it is not clear whether this localization represents trapped soluble 63-kDa protein or reveals a form bound to part of the cortex.

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REFERENCES

1. Kelly RB: Science 230:25–32, 1985.
2. Del Castillo J, Katz B: "Microphysiologie Comparee des Elements Excitables." Coll Int CNRS Paris, No 67:245:58, 1957.
3. Satir BH, Garofalo RS, Otter T, Satir P: In de Bernard B, Sottocasa GL, Sandri G, Carafoli E, Taylor AN, Vanaman TC, Williams RJP (eds): "Calcium-Binding Proteins." Amsterdam: Elsevier, 1983, pp 433–447.
4. Satir BH: In Gilula NB (ed): "Membrane-Membrane Interactions." New York: Raven Press, 1980a, pp 45–58.
5. Lucy JA: Nature 227:815–817, 1970.
6. Hirata F, Axelrod J: Proc Natl Acad Sci 75:2348–2352, 1978.
7. Hirata F, Viveros OH, Diliberto EJ Jr, Axelrod J: Proc Natl Acad Sci 75:1718–1721, 1978.
8. Adelstein RS, Conti MA, Pato MD: Ann NY Acad Sci 356:142–150, 1980.
9. Levitan, IB: J Memb Biol 87:177–190, 1986.
10. Browning MD, Huganir RL, Greengaard P: J Neurochem 45:11–23, 1985
11. DeRiemer SA, Strong JA, Albert KA, Greengaard P, Kaczmarek LK: Nature 313:313–316, 1985.
12. Nishizuka J: Science 233:305–312, 1986.
13. Williamson JR, Cooper RH, Joseph SK, Thomas AP: Am J Physiol 248:C203–C216, 1985.
14. Berridge MJ, Irvine RF: Nature 312:315–321, 1984.
15. Garofalo RS, Gilligan DM, Satir BH: J Cell Biol 96:1072–1081, 1983.
16. Satir B, Schooley C, Satir P: Nature 235:53–54, 1972.
17. Satir BH, Schooley C, Satir P: J Cell Biol 56:153–176, 1973.
18. Beisson J, Lefort-Tran M, Pouphile M, Rossignol M, Satir B: J Cell Biol 69:126–143, 1976.
19. Orias E, Flacks M, Satir BH: J Cell Sci 64:49–67, 1983.
20. Satir BH, Reichman M, Orias E: Proc Natl Acad Sci 83:8221–8225, 1986.
21. Satir BH, Oberg SG: Science 199:536–538, 1978.
22. Garofalo RS, Satir BH: J Cell Biol 99:2193–2199, 1984.

23. Bilinski M, Plattner H, Matt H: J Cell Biol 88:179–188, 1981.
24. Naitoh Y, Kaneko H: Science 176:523, 524, 1972.
25. Naitoh Y, Kaneko H: J Exp Biol 58:657–676, 1973.
26. Otter T, Satir BH, Satir P: Cell Motil 4:249–267, 1984.
27. Kung C: Cold Spring Harbor Conference on Cell Proliferation 941:948, 1976.
28. Satir BH: 38th Annu Proc Elec Micro Soc Am 590–593, 1980b.
29. Satir BH, Garofalo RS, Gilligan DM, Maihle NJ: Ann NY Acad Sci 356:408–409, 1980.
30. Maihle NJ, Dedman JR, Means AR, Chafouleas JG, Satir BH: J Cell Biol 89:695–699, 1981.
31. Garofalo RS, Satir BH: 39th Annu Proc Elec Micro Soc Am 576–579, 1981.
32. Pouphile M, Lefort-Tran M, Plattner H, Rossignol M, Beisson, J: Biol Cell 56:151–162, 1985.
33. Allen RD, Fok AK: Eur J Cell Biol 35:27–34, 1984.
34. Pape R, Plattner H: Eur J Cell Biol 36:38–47, 1985.
35. Aufderheide K: Mol Gen Genet 165:199–205, 1978.
36. Levin RM, Weiss B: Mol Pharmacol 12:581–589, 1976.
37. Busch GR, Satir BH: J Cell Biol 99:383a, 1984.
38. Busch GR, Satir BH: (submitted), 1987.
39. Allen RD, Fok AK: J Cell Biol 97:566–570, 1983.
40. Hopfer U, Lehninger AL, Thompson TE: Proc Natl Acad Sci USA 59:484–490, 1986.
41. Loomis WF, Lippman F: J Biol Chem 172:807, 808, 1948.
42. Leung SK, Fok AK: J Cell Biol 95:A432.
43. Slater EC: J Biochem (Tokyo) 59:392–405, 1955.
44. DeDuve C, DeBarsy T, Poole B, Trovet A, Tulkens P, Van Hoff F: Biochem Pharmacol 23:2495, 1974.
45. Lenz AG, Holzer H: Arch Microbiol 137:104–108, 1984.
46. Matt H, Plattner H, Reichel K, Lefort-Tran M, Beisson J: J Cell Sci 46:41–60, 1980.
47. Gilligan DM, Satir BH: J Cell Biol 97:224–238, 1983.
48. Bayer R, Ehara T: Prog Pharmacol 2:31–37, 1978.
49. Fleckenstein A: Annu Rev Pharmacol Toxicol 17:149–166, 1977.
50. Nowycky MC, Fox AP, Tsien RW: Nature 316:440–443, 1985.
51. Reuter H: Nature 316:391, 1985.
52. Vuoso A, Satir BH: Biophys J 49:200a, 1986.
53. Vuoso A, Satir BH: J Cell Biol 100(5):460a, 1986.
54. Vuoso A, Satir BH: J Protozool 20 (75):14, 1986.
55. Hess P, Lansman JB, Tsien RW: J Physiol (Lond) 358:61P, 1964.
56. Hagiwara S, Byerly L: Annu Rev Neurosci 4:69–126, 1981.
57. Tsien RW: Annu Rev Physiol 45:341–358, 1983.
58. Nelson MT: J Gen Physiol 87(2):201–222, 1986.
59. Vuoso AL, Satir BH: (submitted), 1987.
60. Gilligan DM, Satir BH: J Biol Chem 257:13903–13906, 1982.
61. Zieseniss E, Plattner H: J Cell Biol 101:2028–2035, 985.
62. Murtaugh TJ, Gilligan DM, Satir, BH: J Biol Chem 262:15734–15739, 1987.
63. Gerace, L: TIBS 11:443–446, 1986.
64. Huttner WB, Schiebler W, Greengaard P, De Camilli P: J Cell Biol 96:1374–1388, 1983.
65. Satir BH, Herschenfeld R, Hamasaki T, Murtaugh TJ: Biophys J 51:47a, 1987.
66. Lefort-Tran M, Aufderheide K, Pouphile M, Rossignol M, Beisson J: J Cell Biol 88:301–311, 1981.
67. Beisson J, Rossignol M: Mol Biol Neucleocytoplasmic Relationships 291:1975.
68. Pollack S: J Protozool 21(2):352, 1974.