enter cells. Furthermore, a leupeptin-insensitive alkaline protease also seems to participate in protein turnover in skeletal muscle (*16*).

These results also do not establish that cathepsin B is the only protease inhibited by leupeptin in this tissue. Cathepsin L, an enzyme very similar to cathepsin B and also inhibited by leupeptin, has been found in liver lysosomes (*21*). In addition, muscle contains a soluble alkaline protease, activated by calcium (*5*), which seems sensitive to leupeptin (*16*). These enzymes or perhaps unknown proteases may also contribute to the effects of leupeptin reported here.

These experiments illustrate the value of inhibitors of this class as probes for investigating the functions of proteases in vivo and for elucidating the pathway of protein catabolism. In addition, the results with atrophying muscles provide an experimental rationale for attempting to treat muscle atrophy with leupeptin or other protease inhibitors. Stracher and co-workers (*22*) reported delayed degeneration of cultured cells from normal and dystrophic chick embryo muscles exposed to leupeptin, antipain, and pepstatin. They hypothesized that this was a consequence of decreased protein breakdown. Our results with intact muscles show that leupeptin could indeed act as these workers suggest. Leupeptin may have potential therapeutic uses, since it is apparently nontoxic and is absorbed orally (*23*). However, it may not be an ideal compound for therapeutic use since it is rapidly excreted in the urine and in some species may inhibit other important proteolytic enzymes (such as proteases involved in hemostasis, fibrinolysis, or maturation of secreted proteins) (*23*). Nonetheless, further pharmacological studies with leupeptin and related compounds seem warranted by the results reported here and elsewhere (*22*).

PETER LIBBY
ALFRED L. GOLDBERG
Department of Physiology,
Harvard Medical School,
Boston, Massachusetts 02115

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Paramecium Fusion Rosettes: Possible Function as Ca^{2+} Gates

Abstract. *The function of a specific intramembrane particle array, "the fusion rosette," an essential requirement for exocytosis of trichocysts in* Paramecium, *was probed with a temperature sensitive secretory mutant (nd9). The cells were grown at 27°C, the nonpermissive, nonreleasing temperature at which fusion rosettes do not assemble. Exocytosis could be triggered, nonetheless, by addition of 40 μM ionophore A23187 and 15 mM Ca^{2+} but not Mg^{2+}. Rosette function is bypassed by this procedure, suggesting that during normal release, the rosette acts as a Ca^{2+} channel that allows development of a site-specific increase in Ca^{2+}, which in turn induces fusion and release.*

New information about molecular events involved in the process of vesicular secretion has been obtained with the freeze fracture technique (*1, 2*). The secretory process has been carefully studied in two ciliated protozoa, *Tetrahymena* and *Paramecium*, where the presence of an intramembrane particle array, the fusion rosette, marks the sites toward which the secretory organelles of these cells (mucocysts and trichocysts, respectively) have migrated and docked prior to membrane fusion and release (*2, 3*). The rosette consists of 11 P and E face particles, 15 nm in diameter with one central particle. In *Paramecium*, but not in *Tetrahymena*, the rosette is surrounded by one or two rings of particles (about 7.5 nm in diameter), which play no role in secretion (*4, 5*). An intimate connection between the rosette and secretion was clearly demonstrated with a series of secretory mutants of *Paramecium*. Beisson *et al.* (*4*) showed that a temperature-sensitive mutant, nd9, when grown at the nonpermissive temperature (27°C) neither assembles rosettes nor secretes mature attached trichocysts; however, the same cell grown at the permissive temperature (18°C) both assembles rosettes and regains the normal capacity for secretion.

In many systems, control of secretion appears to be dependent on the presence of Ca^{2+} (*6*). Although the exact role that this ion plays at a molecular level is still unclear, it is postulated that a rise in cytoplasmic free Ca^{2+} is usually necessary for normal stimulus secretion coupling. Secretion can be induced in wild-type *Paramecium* in the presence of extracellular Ca^{2+} by exposure to ionophores such as X-537A or A23187, compounds that facilitate transport of diva-

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lent cations across membranes (*7*). Further, in *Paramecium*, Ca^{2+} has been localized histochemically at the sites of trichocyst attachment (*8*). Using the ionophore A23187 and the temperature-sensitive secretory mutant nd9 of *Paramecium tetraurelia*, we have examined the question of whether the function of the fusion rosette in secretion is related to transport of Ca^{2+}.

We have grown nd9 cells in bacterized 0.25 percent cerophyll, 0.05 percent Na_2HPO_4 at either permissive (18°C) or nonpermissive (27°C) temperatures. Trichocyst release is tested qualitatively in these cells by addition of picric acid (saturated solution) to the medium, the result of which is shown in Fig. 1, a and b, at 18°C and 27°C, respectively. As is well known for these cells, picric acid apparently acts as a nonspecific trigger of massive secretion before the cell is fixed and is used routinely in selection of secretory mutants (*4*). In nd9, at the permissive temperature, secretion occurs as in wild type, and the secretory product (trichocysts), easily visible in the light microscope, can be seen as long thin needles surrounding the entire cell (Fig. 1a). However, at the nonpermissive temperature this halo of trichocysts is completely absent (Fig. 1b).

For standardization of the assay of trichocyst release, the cells, harvested in late log phase, were washed and resuspended in a defined salt solution containing either 15 m*M* $CaCl_2$ or 15 m*M* $MgCl_2$ and 1 m*M* KCl, 0.1 m*M* EDTA, and 5 m*M* tris-HCl (*p*H 7.4) at a cell concentration of about 10,000 cells per milliliter; the cells were tested for release after being resuspended in this solution from 0 to 2 hours. When trichocyst release was again induced by the addition of picric acid to $CaCl_2$-containing medium, release of about 40 percent of total trichocysts present per cell occurs in nd9 cells grown at the permissive temperature (18°C) (Table 1) (*9*), an amount not significantly different from the amount of discharge obtained previously. When $MgCl_2$ was substituted for $CaCl_2$, exocytosis was inhibited (Table 2). Further, nd9 cells grown at 27°C did not release with picric acid in either the Ca^{2+}- or Mg^{2+}-containing solutions (Table 2).

If the effect of picric acid addition in producing trichocyst discharge is primarily to open Ca^{2+} channels so that an increase in local Ca^{2+} concentration occurs at the trichocyst site, then (i) the inhibition of exocytosis by Mg^{2+} and (ii) ionophore substitution in the presence of Ca^{2+} for picric acid stimulation could readily be explained. As a test for this, A23187 was dissolved in dimethyl sulfoxide and added to the standard test solution containing either 15 m*M* $CaCl_2$ or $MgCl_2$ to a final concentration of 40 μM ionophore in 5 percent dimethyl sulfoxide. In solvent controls we omit the ionophore.

Table 1. Quantitation of release in nd9.

Temperature (°C)	Treatment	Trychocyst release per cell (No. ± S.D.)
18	Picric acid + medium	205 ± 79*
	Picric acid + Ca^{2+}	167 ± 86*
	A23187 + Ca^{2+}	92 ± 59
27	A23187 + Ca^{2+}	67 ± 21

*Not significantly different (Student's *t*-test, $P \sim .15$)

Table 2. Relative release of trichocysts in nd9 after various treatments. Picric acid experiments provide a count of standard halo around fixed cells. Ionophore and dimethyl sulfoxide experiments provide a count of quiescent living cells over a 15-minute period. Comparative values are based on samples from the same culture. At least 30 cells were counted for each value.

Treatment	Percent of standard release at	
	18°C	27°C
Picric acid + Ca^{2+}	100*	0.2
Picric acid + Mg^{2+}	0.4	0.1
A23187 + Ca^{2+}	30	66
A23187 + Mg^{2+}	1.7	5
DMSO + Ca^{2+}	0.7	0.0
DMSO + Mg^{2+}	0.3	1.0

*The value 100 percent is equal to 76 ± 7 (standard error) trichocysts counted for picric acid plus Ca^{2+}.

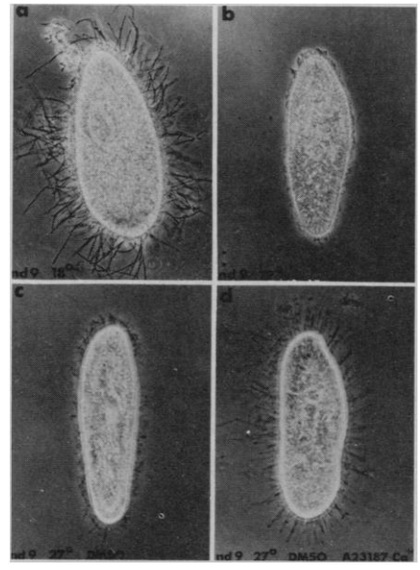

Fig. 1. Phase contrast micrographs of trichocyst discharge and controls in nd9 cells. (a) Normal release response after addition of picric acid to cell grown at 18°C. The halo of released trichocysts (thin needles) surrounds the cell body. The cells were in cerophyll growth medium. (b) An nd9 cell grown at nonpermissive temperature (27°C). No release occurred on addition of picric acid. (c) Ionophore solvent control. Cells were grown at 27°C and placed in defined salt solution containing Ca^{2+}, and at a final concentration of 5 percent dimethyl sulfoxide. (d) Release response triggered in nd9 cells grown at 27°C on addition of A23187. The conditions were identical to those in (c), except for the addition of 100 μM A23187.

At the permissive temperature (18°C) in the presence of Ca^{2+}, as was expected, A23187 substituted for picric acid and stimulated exocytosis. About 10 to 20 percent of total trichocysts were discharged, about half of that observed with picric acid stimulation (Table 1). Although discharge was greatly inhibited when Mg^{2+} was substituted for Ca^{2+} in these experiments, some discharge (1 to 2 percent of total trichocysts) was still seen (Table 2). This result suggests that some Ca^{2+} may have been available from internal sources when ionophore was added. Under our conditions, the presence of solvent (DMSO) alone did not trigger discharge with either Ca^{2+} or Mg^{2+}.

At the nonpermissive temperature (27°C), an unexpected result was obtained. Mass discharge of trichocysts was still induced by the presence of 40 μM ionophore and Ca^{2+} (Tables 1 and 2). The effect of ionophore on discharge at 27°C is shown dramatically in Fig. 1, c and d. Here we have increased the ionophore concentration to 100 μM in order to retain the secreted trichocysts around the cell body for direct comparison to the picric acid results (Fig. 1b). This concentration of ionophore was lethal. Figure 1c represents the solvent control (no discharge) and Fig. 1d demonstrates discharge in the presence of ionophore and Ca^{2+}. Therefore, the ionophore A23187 in the presence of Ca^{2+} apparently phenocopies the fusion rosette in this mutant in ability to induce exocytosis.

Light and transmission electron micrographs of cells exposed to ionophore or solvent (or both) under the various conditions showed no major morphological differences between discharging and nondischarging cells other than changes in the number of released trichocysts. In particular, trichocyst attachment was

normal, and no increase in intracellular release of trichocysts was observed. Although no information is yet available for *Paramecium*, freeze fracture micrographs of ionophore-induced mucocyst discharge in *Tetrahymena* (*10*) indicates that discharge occurs via the normal sequence of membrane fusion processes.

We envision the sequence as follows. When the cell is stimulated at its external surface, a transmembrane event at the site of the fusion rosette is triggered. The simplest explanation of this event would be that the rosette particles act as Ca^{2+} channels that open upon stimulation. A site-specific increase in Ca^{2+} between the two competent partner membranes would then induce membrane fusion and subsequent exocytosis (*11*). At 18°C in nd9 cells, in the presence of Ca^{2+} picric acid would stimulate exocytosis. In the presence of external Mg^{2+} rather than Ca^{2+}, no Ca^{2+} would be available and exocytosis would be blocked. At 27°C, where the fusion rosette is missing, with an external stimulus (that is, picric acid) exocytosis cannot take place even where Ca^{2+} is present in the medium. Ionophore addition would substitute for the rosette by providing the necessary Ca^{2+} pathway across the membrane.

BIRGIT H. SATIR,
STEPHEN G. OBERG
Department of Anatomy, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461, and *Department of Physiology-Anatomy, University of California, Berkeley 94720*

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Double-Stranded RNA Is Present in Extracts of Tobacco Plants Infected with Tobacco Mosaic Virus

Abstract. *Serologically specific electron microscopy was used to detect double-stranded RNA in extracts of tobacco infected with tobacco mosaic virus. Assays were made immediately after extraction, without purification, concentration, or treatment with phenol or detergent. This indicates that the double-stranded RNA is native and is not an artifact induced by purification methods.*

The possible presence of double-stranded RNA in tissues infected with viruses that have single-stranded RNA genomes has been reviewed (*1*). RNA complementary to parental viral RNA is present in these tissues and is considered to function as a template for the synthesis of viral RNA. Evidence has been given to show that these complementary strands of RNA are double-stranded and, in contradiction, they are probably only loosely bound in short segments at the point of synthesis. The case for double-stranded RNA is supported by experiments showing that it can be isolated from virus-infected tissue and from viral replicase systems in vitro. Methods for isolating double-stranded RNA include treatment with phenol and detergent, which remove proteins from nucleic acids; under such conditions complementary strands tend to become double-stranded. Double-stranded RNA is indicated by resistance to ribonuclease and, after sufficient purification and concentration, by biochemical characterization or examination with the electron microscope. Arguments against the existence of double-stranded RNA include experiments showing that very little ribonuclease-resistant RNA is found in infected tissue extracts or replicase systems when they are not treated with phenol or detergent. An immunofluorescent technique was used to suggest the presence of double-stranded RNA in the cytoplasm of cells infected with sindbis virus (*2*). Since as few as five nucleotide pairs may be sufficient to provide a binding site for antibodies, this technique is

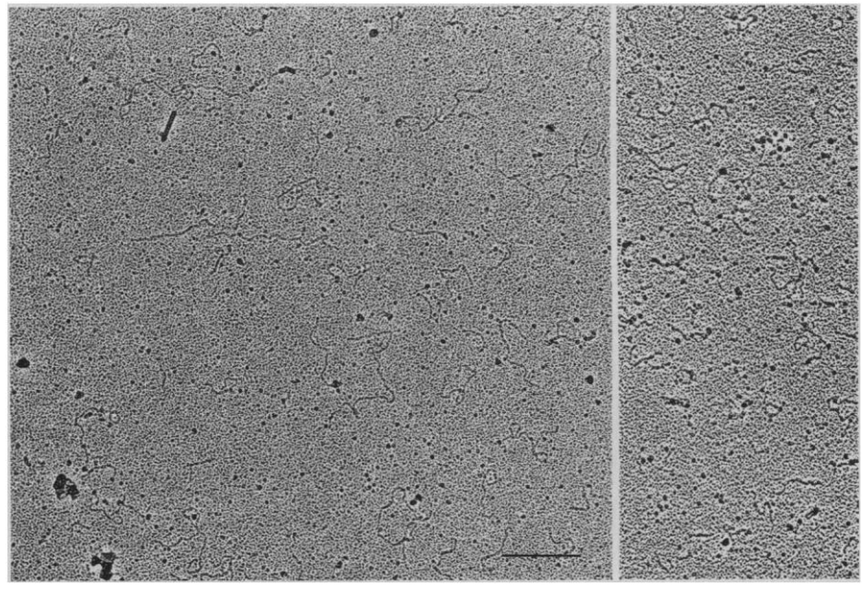

Fig. 1. Electron micrographs showing double-stranded RNA from an extract of tobacco leaf infected with TMV (left) and from a solution containing double-stranded polyinosinic · polycytidylic acid (2 μg/ml) (right). Samples of tobacco leaves inoculated with TMV were collected at intervals after inoculation and stored at −20°C. Double-stranded RNA was readily detected 40 hours after inoculation. The result shown is from a sample collected 4 days after inoculation. Filmed electron microscope grids were floated for 30 minutes on antiserum to double-stranded RNA (diluted 1 : 1000 with 0.05*M* tris-HCl, *p*H 7.2). The grids were washed with this tris buffer and placed on dilutions of synthetic double-stranded RNA, or on extracts of leaf tissue prepared by grinding 60 mg of frozen tissue with 0.54 ml of the tris buffer containing 0.4*M* sucrose and 0.15*M* NaCl (*10*). After 2 hours, the grids were washed with the tris buffer and placed on cytochrome c (0.1 mg/ml) in tris buffer for 15 minutes. The grids were then washed with distilled water, dipped for 30 seconds in $5 \times 10^{-5}M$ uranyl acetate and $5 \times 10^{-5}M$ HCl in 95 percent ethanol, rinsed with 95 percent ethanol, dried in air, and rotary-shadowed with platinum-palladium (*5*) (scale bar, 500 nm).

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