Selectivity of Intracellular Proteolysis: Protein Substrates Activate the ATP-Dependent Protease (La)

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A critical enzyme in protein breakdown in *Escherichia coli* is protease La (the *lon* gene product), which hydrolyzes proteins and adenosine triphosphate (ATP) in a coupled process. The mechanism of this process was studied with fluorogenic tripeptides. Although proteins and peptides are degraded at the same active site, protein substrates enhance the ability of the enzyme to degrade these peptides two- to tenfold. Proteins that are not substrates had little or no effect. Thus, protein substrates must bind to protease La at two sites, the active site and an allosteric site whose occupancy enhances proteolytic activity. This effect did not require that the proteins themselves be degraded. Proteins could induce peptide breakdown even in the absence of ATP, and proteins and ATP had additive effects in stimulating peptidase activity. A multistep cyclical mechanism is proposed in which the binding of the substrate and ATP activates the protease. The enzyme can then cleave a peptide bond, but is inactivated through ATP hydrolysis. Such a mechanism may help account for the selectivity of protein breakdown and prevent inappropriate or excessive proteolysis in vivo.

ANIMAL AND BACTERIAL CELLS DEgrade particularly rapidly proteins with highly abnormal conformations, which may result from mutations, biosynthetic errors, postsynthetic damage, or genetic engineering (*1–3*). The basis of this selectivity and the mechanisms preventing excessive proteolysis in vivo are not clear. Another feature of intracellular protein degradation is that it requires metabolic energy (*1–3*). In *Escherichia coli* and in mitochondria, this requirement results from the involvement of a new type of proteolytic enzyme, which hydrolyzes proteins and adenosine triphosphate (ATP) in a coupled process (*1, 4–8*). The ATP-dependent protease in *E. coli*, protease La, is encoded by the *lon* gene (*5, 7*). It is an unusually large protease comprised of four identical 94,000-dalton subunits and catalyzes the rate-limiting steps in the breakdown of most abnormal and certain normal proteins (*4, 5, 7–13*).

The involvement of ATP hydrolysis in the function of a protease poses intriguing mechanistic and physiological questions. We have found that in the presence of ATP and divalent cations protease La can degrade various small hydrophobic peptides (*14*). Our studies (*15*) indicate that cleavage of these peptides represents a partial reaction that can be dissociated from other steps necessary for ATP-dependent breakdown of large proteins. Unlike the degradation of proteins, the hydrolysis of these oligopeptides requires only binding of ATP to the enzyme and does not require cleavage of the high energy bond. Nonhydrolyzable ATP analogs support the breakdown of these peptides, but not that of large proteins. Moreover, inhibition of the adenosinetriphosphatase (ATPase) function prevents protein breakdown, but does not inhibit the hydrolysis of small peptides. We have therefore proposed (*15*) a cyclical model for protease La action in which (i) ATP initially binds to the inactive protease and allosterically activates peptide bond cleavage; (ii) the hydrolysis of ATP to adenosine diphosphate (ADP) occurs subsequently, and this process is stimulated by protein substrates (*8*); (iii) the enzyme should be temporarily inactive until a new ATP binds to it. Presumably with large proteins, this cycle would be repeated until small acid-soluble peptides are generated.

In studying the relative preference of the enzyme for proteins or peptide substrates, we unexpectedly found that the interaction of protein substrates with the protease increases its ability to hydrolyze peptide bonds. We systematically examined the mechanism of this activation by substrates, since it may help account for the specificity and regulation of intracellular protein breakdown.

We previously showed that hydrolysis of several fluorogenic tripeptide substrates (for example, glutaryl-Ala-Ala-Phe-methoxynaphthylamine (MNA) (Glt, glutaryl) (*14*), which is cleaved between the phenylalanine and the fluorescent MNA group) occurs at the same active site on the protease as protein cleavage (*14*). Thus, these small peptides, when added together with protein substrates, inhibit the degradation of the proteins. Since protease La shows a much higher affinity for protein substrates (*8, 16*) than for these peptides (K_m of 300 to 500 μM) (*14*), we anticipated that casein (K_m of 20 to 50 μM) or other protein substrates would cause a marked inhibition of the hydrolysis of these peptides. However, when present together, proteins were found to stimulate the ATP-independent breakdown of Glt-Ala-Ala-Phe-MNA severalfold (for example, Fig. 1 and Tables 1 and 2). Casein over a broad range of concentrations (0.1 to 10 μM) increased peptide hydrolysis, even when the peptide was present at much higher molar concentrations (500 μM). Half-maximal stimulation occurred with ^{3}H-labeled casein at concentrations between 6 and 12 μg/ml.

Since the casein did not inhibit peptide hydrolysis even at concentrations far above its K_m, it must be activating hydrolysis of fluorogenic peptides by binding to a site distinct from the active site. In contrast, binding of protein substrates did not enhance degradation of other proteins present simultaneously. For example, denatured bovine serum albumin (BSA), which is a substrate for protease La, stimulated peptide hydrolysis two- to sixfold (Table 1) though it inhibited the breakdown of ^{3}H-labeled casein slightly (14 percent), as expected for

Table 1. Casein can activate hydrolysis of Glt-Ala-Ala-Phe-MNA and ^{125}I-labeled insulin in the absence of ATP. Protease La (1.5 μg) was incubated with 500 μM Glt-Ala-Ala-Phe-MNA in the presence of 10 mM Mg^{2+} at 37°C (*14*). Additions included either 0.5 mM ATP or 20 μg of α-casein. Each of these experiments involved a different preparation of the protease and illustrate the additive or in certain preparations (experiments 3 and 4) synergistic effects of casein and ATP. ^{125}I-labeled insulin chains were generated by treatment of ^{125}I-labeled insulin with dithiothreitol. The breakdown ^{125}I-labeled insulin chains (20 μg) to acid-soluble material was assayed in a reaction volume of 200 μl containing 1 μg of protease La, 50 mM tris-HCl (*p*H 8), 10 mM Mg^{2+}, and 1 mM dithiothreitol. After 1 hour, trichloroacetic acid was added (15 percent final concentration) and BSA (2 mg/ml). After centrifugation, the supernatant was counted in a gamma spectrometer.

Addition	Hydrolysis			
	Glt-Ala-Ala-Phe-MNA (pmol/hour)			[^{125}I]Insulin chains (μg/hour)
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
None	18	52	0	0
Casein	159	182	45	0.09
ATP	143	314	125	0.09
ATP + casein	325	624	600	0.59

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alternative substrates. Similarly, glucagon, another good polypeptide substrate, weakly inhibits casein hydrolysis (by 10 percent), but activated Glt-Ala-Ala-Phe-MNA hydrolysis fourfold (Fig. 2).

Preparations of protease La that are purified and stored (Table 1) (*14*) generally show low peptidase activity with Mg^{2+} present, and the addition of ATP stimulates this process 8- to 20-fold. Even in the absence of ATP, the addition of casein stimulated peptide hydrolysis. Thus, the binding of protein substrates to protease La does not require ATP. Furthermore, when ATP and casein were both included in the assay mixture, their effects on Glt-Ala-Ala-Phe-MNA hydrolysis were additive (Table 2) or in some preparations synergistic (Table 1). The additivity of these maximal effects suggests that proteins and ATP enhance the peptidase reaction by distinct mechanisms. With different enzyme preparations, the relative degrees of stimulation of peptide hydrolysis by casein and by ATP varied (Table 1). In some, there was no basal peptide hydrolysis, but addition of casein or ATP induced this activity (Table 1).

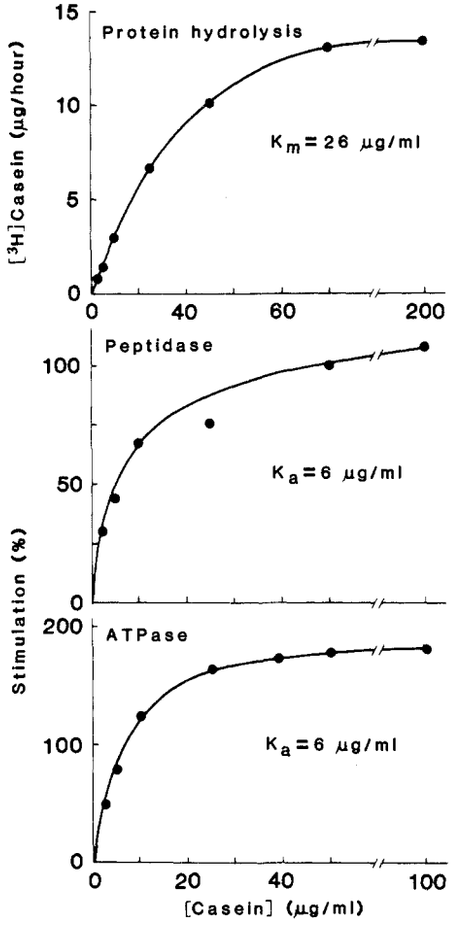

Fig. 1. The effect of casein concentration on the hydrolysis of Glt-Ala-Ala-Phe-MNA, ATP, and casein itself by protease La. The degradation of fluorogenic peptides was measured by following the release of methoxynaphthylamine (MNA) from Glt-Ala-Ala-Phe-MNA (*14*). The assay mixtures were held for 1 hour at 37°C and included 0.5 m*M* ATP, 500 μ*M* peptide, 1.5 μg of protease La, and increasing amounts of α-casein in a final volume of 200 μl. ATP hydrolysis in 30 minutes was measured as described (*8*). The hydrolysis of ^{3}H-labeled casein was assayed at 37°C at intervals up to 1 hour to ensure the linearity of the assay. The formation of acid-soluble peptides was determined by liquid scintillation counting after addition of 575 μl of 10 percent trichloroacetic acid and 25 μl of 10 percent BSA (*8*). Higher concentrations of casein did not further enhance these processes. Protease La was purified to homogeneity (*5, 16*) from frozen *E. coli* containing the plasmid PJMC40 which carries the *lon* gene (*26*).

This induction of peptidase activity by casein in the absence of nucleotide must mean that this effect does not require the breakdown of this protein. Accordingly, vanadate, which inhibits ATP hydrolysis and decreases in parallel the degradation of ^{3}H-labeled casein (*8*), had no effect on the ability of casein to stimulate Glt-Ala-Ala-Phe-MNA hydrolysis (Table 3). Furthermore, the stimulation of peptide hydrolysis by casein still occurred in the presence of various nonhydrolyzable ATP analogs (Table 3), which do not support breakdown of proteins (*5, 8, 15*). Like ATP (Table 1), these nonmetabolized analogs had additive effects with casein in activating peptide hydrolysis.

Protein substrates also stimulated hydrolysis of other peptides. ATP or casein alone caused little degradation of free insulin chains by protease La, but the addition of ATP and casein together had a synergistic effect in activating breakdown of this substrate (Table 1). Similar effects occurred with other fluorogenic peptide substrates. Protease La has some similarities to chymotrypsin in its preference for hydrophobic substrates (*14*). This enzyme cleaves Suc-Phe-Leu-Phe-MNA (Suc, succinyl) rapidly, and this process also was stimulated threefold by casein. In addition, several other peptides (such as Glt-Ala-Ala-Ala-MNA or Glt-Gly-Gly-Phe-MNA), which are much poorer substrates than Glt-Ala-Ala-Phe-MNA (*14*), were hydrolyzed three- to fourfold more rapidly on addition of casein. However, CBZ-Ala-Arg-Arg-MNA, which is a substrate for trypsinlike enzymes, but not for protease La (*14*), was not hydrolyzed even in the presence of casein. Thus, proteins seem to enhance the hydrolysis of both good and poor substrates similarly, without changing the apparent specificity of the active site. These experiments suggest that casein increases the V_{max} for peptide substrates, but a kinetic analysis of this issue was impossible because of the insolubility of these hydrophobic peptides at high concentrations and the inhibitory effect of organic solvents (*14*).

The ability of other protein substrates to stimulate peptide hydrolysis is shown in Table 2 and Fig. 3. Both denatured BSA and glucagon were able to enhance the hydrolysis of Glt-Ala-Ala-Phe-MNA. Like α-casein

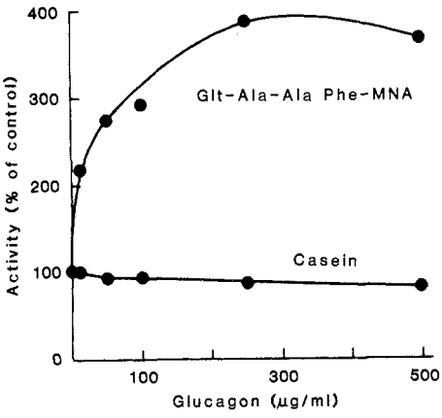

Fig. 2. The effect of glucagon on the hydrolysis of Glt-Ala-Ala-Phe-MNA and ^{3}H-casein by protease La. Protease La (1.5 μg) was held for 1 hour at 37°C with 20 μg of ^{3}H-labeled casein (100 μg/ml) (*8*) or with Glt-Ala-Ala-Phe-MNA (500 μ*M*) in the presence of increasing amounts of bovine glucagon.

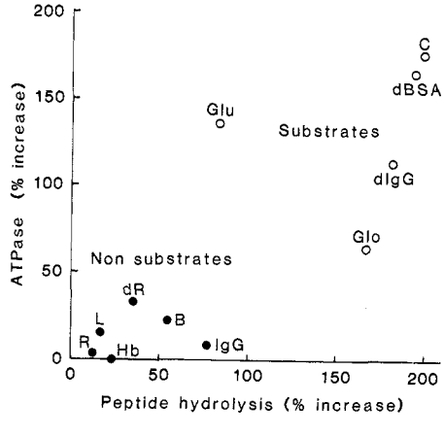

Fig. 3. The effect of substrates and nondegraded proteins on the hydrolysis of Glt-Ala-Ala-Phe-MNA and ATP by protease La. The hydrolysis of Glt-Ala-Ala-Phe-MNA (500 μ*M*) in the presence or absence of a variety of proteins was carried out with 1.5 μg of protease La for 1 hour at 37°C (*14*). Similarly, the hydrolysis of ATP was measured in the presence and absence of these proteins by a colorimetric assay (*27*) as described (*8*). Proteins were denatured by reduction and alkylation with iodoacetamide in 6*M* guanidine-HCl followed by dialysis against water. Protein concentrations were estimated by A_{280} and compared with reported extinction coefficients for each. The abbreviations for the different proteins and their denatured forms are: L, lysozyme; C, casein; Glu, glucagon; Hb, hemoglobin; R, ribonuclease; dR, denatured ribonuclease; B, BSA; dBSA, denatured BSA; IgG, immunoglobulin; dIgG, denatured IgG; Glo, globin.

Table 2. Ability of polypeptide substrates to stimulate hydrolysis of Glt-Ala-Ala-Phe-MNA by protease La. The assay mixture contained 0.5 m*M* Glt-Ala-Ala-Phe-MNA, 1 m*M* ATP, 10 m*M* Mg^{2+}, and 20 μg of the protein substrates. Incubations were for 1 hour at 37°C. BSA was reduced and carboxymethylated to make it susceptible to proteolysis (*8*).

Addition	Fluorescence units	
	−ATP	+ATP
None	0.57	3.60
Casein	3.10	6.54
Globin	2.69	6.38
Denatured BSA	1.48	5.14
Glucagon	1.56	4.70

(Table 3), these proteins caused a stimulation both in the presence and in the absence of ATP, where the proteins themselves are not degraded (*4, 5, 7, 8*). Only those proteins that are degraded to acid-soluble fragments stimulated the hydrolysis of Glt-Ala-Ala-Phe-MNA twofold or more.

By contrast, proteins that are not substrates, such as ribonuclease or lysozyme, are relatively ineffective at enhancing peptide activity. However, several proteins that were not substrates, such as native BSA or immunoglobulin G (IgG), and did not promote peptide hydrolysis, were degraded and activated peptide cleavage after complete denaturation (by reduction and carboxymethylation in guanidine-hydrochloride). Similarly, native hemoglobin had little or no effect, but after extraction of the heme moiety, which partially denatures the protein (*17*), the globin became a substrate (*4*) and stimulated peptide hydrolysis (Table 2 and Fig. 3). Ribonuclease and lysozyme are poor substrates even after reduction and carboxymethylation and do not stimulate peptidase activity significantly. Thus, merely denaturing a protein does not appear sufficient for enzyme activation or proteolytic susceptibility.

Previously, we found that proteins that are substrates for protease La stimulate ATPase activity severalfold, while polypeptides which are not degraded do so only poorly or not at all (*8*) (Fig. 1). Furthermore, this rise in ATPase activity is directly proportional to the number of peptide bonds cleaved in the protein (*8, 16, 18*). We therefore looked to see whether proteins that stimulate peptide hydrolysis also promote ATP breakdown by protease La. These two properties of proteins correlated well with each other (Fig. 3). Those polypeptides and denatured proteins that were substrates enhanced peptidase and ATPase activities to a similar extent. By contrast, proteins not degraded to acid-soluble fragments (native BSA, native IgG, or hemoglobin) were relatively poor or incapable of stimulating ATP breakdown (Fig. 3).

To test whether this allosteric activation occurs normally during protein degradation, we compared in a single experiment the concentrations of ^{3}H-labeled casein that give maximal stimulation of the peptidase and the ATPase and maximal rates of ^{3}H-labeled casein degradation. The K_a for stimulation of the ATPase and of peptide hydrolysis are indistinguishable (6 μg/ml) (Fig. 1). The agreement between these values is further evidence that binding of a protein to a single regulatory site is essential for both allosteric effects. The K_m for casein hydrolysis appeared equal or somewhat higher (26 μg/ml) than the Ka for these allosteric effects. If the K_m and K_a were equal, it would suggest that binding to a single allosteric site initiates all three processes. A higher value for the K_m would suggest that the protein binds first to the allosteric site and then the active site, whose affinity is somewhat lower. In either case, the allosteric effects appear to be fully operative during ATP-dependent proteolysis.

The present study reveals an important new feature of the ATP-dependent protease, its activation by protein substrates. To cause this two- to tenfold enhancement of proteolytic activity, protein substrates must interact with a regulatory site on the enzyme that is distinct from the active site; otherwise, they would cause a competitive inhibition of peptide hydrolysis. Thus, protease La must contain two recognition sites for protein substrates: (i) the active site, which has a preference for hydrophobic sequences (*14*), and (ii) a regulatory site that somehow can recognize whether proteins are unfolded (Fig. 3). It is unclear whether this allosteric site also prefers certain amino acid sequences or whether it responds only to general conformational features of the potential substrate. In either case, both sites probably contribute to the ability of protease La to digest selectively highly abnormal proteins and certain normal polypeptides (*19, 20*).

Table 3. The breakdown of ATP or casein is not required for the stimulation by casein of Glt-Ala-Ala-Phe-MNA cleavage by protease La. Assays were for 1 hour at 37°C and included 1 μg of protease La, 0.5 m*M* nucleotide, 20 μg of α-casein, and 250 μ*M* Glt-Ala-Ala-Phe-MNA. Casein degradation to acid-soluble material did not occur with AMPPCP (β,γ-methyleneadenosine 5′-triphosphate, or AMPPNP (β,γ-imidoadenosine 5′-triphosphate (*8*), and was greatly inhibited in the presence of ATP plus 1 m*M* vanadate (*8*) or AMPCPP (α,β-methyleneadenosine 5′-triphosphate (*5*) compared to rates with ATP.

Nucleotide	Peptide hydrolyzed (pmol/hour)	
	−Casein	+Casein
None	20	158
ATP	150	333
ATP + vanadate	165	280
AMPPCP	87	245
AMPPNP	116	303
AMPCPP	270	403

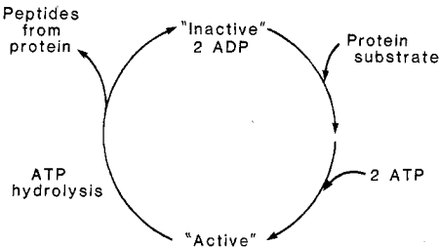

Fig. 4. Proposed mechanism of ATP-dependent protein breakdown. This model implies that degradation of large proteins to peptides involves multiple rounds of this reaction cycle, which was elucidated with the use of peptide substrates to monitor the enzyme's proteolytic activity. This proposal is based on the following observations obtained with peptide substrates. (i) Proteins and ATP activate allosterically peptide hydrolysis; (ii) ATP hydrolysis is not required for cleavage of peptide bonds (*15*); (iii) hydrolysis of ATP to ADP inhibits peptide bond cleavage (*14*); (iv) two ATP molecules are consumed for each peptide bond hydrolyzed in proteins (*18*).

This substrate-induced activation must take place as part of the enzyme's normal functioning because only potential substrates elicit this allosteric effect, and because casein caused half-maximal stimulation of peptide hydrolysis at concentrations that are similar to or lower than the K_m for casein degradation (Fig. 1). Therefore, the activation induced by binding of a potential substrate to the regulatory site probably is an initial event triggering the proteolytic process. Accordingly, proteins can induce peptide hydrolysis without being degraded themselves (Tables 1 to 3). Thus, the binding of potential substrates must induce a conformational change which also increases the ATPase activity (Figs. 1 and 3), and causes other functional changes in the enzyme (*21*). Peptidase activity increases upon binding of a protein or ATP (Tables 1 and 2), both of which are substrates as well as allosteric activators. Since they have additive effects, they must be inducing distinct structural alterations in the enzyme.

With the binding of a substrate and four ATP molecules (*18*), the enzyme's capacity for peptide bond cleavage is enhanced maximally as is nucleotide hydrolysis (Figs. 1 and 3). This protein-induced ATPase may be important in catalysis, for example, allowing the enzyme to act processively on the substrate (*15*); but it also yields ADP, a potent inhibitor (*14*). Recently, Menon and Gold-

berg found that protein substrates also cause release of bound ADP (*21*). Repetition of this cycle of activation by nucleotide binding and inactivation through ATP hydrolysis should eventually generate small peptides from large proteins. For each cycle, however, two criteria must be fulfilled by the protein substrate for continued proteolysis: a hydrophobic sequence (*14*) in the active site and occupancy of the regulatory region. Thus, by binding to both sites, a single protein can induce its own destruction, and should not affect the degradation of other proteins (Fig. 2).

The existence of proteases in the cytosol should be highly damaging to the cell unless precise mechanisms or inhibitors exist to limit their activity. Our findings suggest a mechanism by which intracellular protease activity may be controlled. Even if protease La is free in the cytosol, it should be relatively inactive, unless it binds to an appropriate substrate, and denatured proteins seem to be preferred activators of the protease (Fig. 3). This activation mechanism thus may have evolved to increase selectivity and to prevent inappropriate hydrolysis of normal cell proteins. The specificity of protein breakdown in part depends on which proteins bind to and activate protease La. This activation mechanism seems analogous to the regulation of tissue plasminogen activator, which is inactive until it binds to its substrate—a fibrin clot (*22*).

An important further feature of protease La action is its repeated inactivation through ATP hydrolysis, which means that proteolysis ceases after each endoproteolytic round is completed. Thus, the ATPase should help ensure that excessive proteolysis does not occur, after the enzyme has been activated.

This enzyme mechanism thus may represent a kinetic method for isolation of a potentially dangerous enzymatic activity. Presumably, the same selective pressures that favored the sequestration of cellular proteases in lysosomes or hydrolytic vacuoles in eukaryotic cells would have favored careful kinetic regulation of this critical cytosolic protease. These novel regulatory features can probably be found in other enzymes. Like bacteria, mitochondria contain an ATP-dependent pathway for protein breakdown (*23*) and an ATP-hydrolyzing protease that resembles protese La (*6*); it also shows a stimulation of peptidase and ATPase activities by protein substrates (*6*). Perhaps an analogous mechanism involving activation by proteins and inactivation through ATP hydrolysis may also help prevent inappropriate or excessive proteolysis in the ATP-dependent pathways in the mammalian cytosol (*3, 24, 25*).

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Restoration by Calmodulin of a Ca^{2+}-Dependent K^+ Current Missing in a Mutant of *Paramecium*

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A combination of genetics, biochemistry, and biophysics was used to show that calmodulin is involved in the regulation of an ion channel. Calmodulin restored the Ca^{2+}-dependent K^+ current in *pantophobiac*, a mutant in *Paramecium* that lacks this current. The restoration of the current occurred within 2 hours after the injection of 1 picogram of wild-type calmodulin into the mutant. The current remained for approximately 30 hours before the mutant phenotype returned. The injection of calmodulin isolated from *pantophobiac* had no effect. These results imply that calmodulin is required for the function or regulation of the Ca^{2+}-dependent K^+ current in *Paramecium*.

THE EXCITABLE MEMBRANE contains ion channels that control the flow of ions into and out of the cell. The factors that regulate these ion channels are poorly understood. Calcium-dependent K^+ channels are activated by voltage and cytoplasmic Ca^{2+}, and they exert a control on various Ca^{2+}-regulated functions of the cells through membrane repolarization (*1, 2*). The molecular mechanisms by which this channel is regulated are unknown. Single-channel conductance and toxin sensitivity distinguish several types of Ca^{2+}-dependent K^+ channels (*3*). A Ca^{2+}-dependent K^+ current of the unicellular *Paramecium tetraurelia* has been studied macroscopically by means of a whole-cell voltage-clamp technique (*4, 5*) and microscopically in inside-out patches excised from surface membrane (*6*). The macroscopic Ca^{2+}-dependent K^+ current in *Paramecium* is almost entirely missing in a class of mutants called *pantophobiac* (*5*). Since this current participates in shutting off the Ca^{2+} excitation, its loss prolongs membrane excitation and exaggerates locomotor responses to stimuli (*5*). We showed earlier that the behavioral defect of two of these mutants (*pntA* and *pntB*) can be corrected by the microinjection of cytoplasm from wild-type *Paramecium* (*7*). We now report that the factor that restores the Ca^{2+}-dependent K^+ current is calmodulin.

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