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Proteolytic Activation of a Hyperpolarization- and Calcium-Dependent Potassium Channel in *Paramecium*

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Summary. The effects of proteolysis on a hyperpolarization- and Ca^{2+}-dependent K channel from the surface membrane of *Paramecium tetraurelia* were examined in the inside-out excised patch mode. Treatment with trypsin, pronase or thermolysin removed the Ca^{2+}-dependence of the channel activation, yielding an increase in channel activity greater than 2.5-fold at all Ca^{2+} concentrations between 10^{-4} and 10^{-8} M. Thermolysin additionally removed the voltage dependence of channel opening and gave the most activation among the three proteases tested. Proteolysis did not affect the single-channel conductance. In an analogy to the mechanism of activation of many Ca^{2+}-dependent enzymes it is suggested that this *Paramecium* channel has a cytoplasmic inhibitory domain which can be removed by proteolysis, and that the physiological activation by Ca^{2+} is due to a temporary removal of this inhibition. Moreover, these findings indicate structural differences between depolarization-, Ca^{2+}-dependent K channels (BK channels) and the hyperpolarization-, Ca^{2+}-dependent K channels in *Paramecium*.

Key Words *Paramecium* · patch clamp · K channel · Ca^{2+} dependence · proteolysis

Introduction

There are two major classes of Ca^{2+}-dependent K channels known to date: large conductance (>150 pS) BK channels and small conductance (<20 pS) SK channels (Blatz & Magleby, 1986, 1987). The BK channels are activated by depolarization and blocked by TEA. The SK channels, blocked by apamin, are only weakly voltage-dependent and are more active upon hyperpolarization. In addition to BK and SK channels there are other Ca^{2+}-activated K channels with an intermediate conductance (4–60 pS), whose properties vary depending on the type of cells (Blatz & Magleby, 1987). Single-channel recordings from *Paramecium* plasma membrane have identified a K channel, which is activated by Ca^{2+} and negative voltage (Saimi & Martinac, 1989). This K channel has a 70-pS conductance in symmetrical 100 mM KCl solution. Thus, this *Paramecium* channel does not seem to belong to either BK channels or SK channels.

Proteases have been shown to modify gating properties of a variety of ion channels. Proteolytic digestion of ion channels is based on molecular conformation of the channel and may provide information on the channel structure. Pronase (Rojas & Armstrong, 1971; Armstrong, Bezanilla & Rojas, 1973) or trypsin (Sevcik & Narahashi, 1975) removes Na channel inactivation in squid axon. Alkaline proteinase *b*, a component of pronase from *Streptomyces*, reduces the voltage dependence of voltage-gated K channel from the sarcoplasmic reticulum (Miller & Rosenberg, 1979). Papain or a protein modifying reagent N-bromoacetamide (NBA) selectively removes inactivation of voltage-dependent K channel in GH_3 cells (Matteson & Carmeliet, 1988). Ca^{2+}-dependent K channels (BK channels) from rat skeletal muscle (Pallotta, 1985) and from cultured medullary thick ascending limb cells (Cornejo, Guggino & Guggino, 1987) were also treated with NBA, and it was shown that both channel activity and Ca^{2+} sensitivity of these channels are reduced by NBA treatment.

We have used trypsin, pronase and thermolysin to characterize further Ca^{2+}- and voltage-dependent gating properties of the *Paramecium* K channel. Trypsin is very specific and cleaves only those peptide bonds at a lysine or an arginine residue. Pronase has a very broad spectrum, and digests many proteins to free amino acids. Thermolysin preferentially reacts with nonpolar amino acids, unlike trypsin. We found that all these agents caused a loss of Ca^{2+} sensitivity of the channel and an increase in channel activity even at low Ca^{2+} concentrations. This finding suggests that the *Paramecium* K channel is structurally different from the BK channels.

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Materials and Methods

Cell Culture

Paramecium tetraurelia cells (a trichocyst-nondischarge mutant, *nd-6*) were grown at 31°C in bacterized casaminoacid culture media similar to those described in the previous paper (Saimi & Martinac, 1989). All experiments were performed at room temperature (20–23°C).

Experimental Procedure

The experimental procedure was the same as that described by Saimi and Martinac (1989). After seal formation, the membrane patch was excised from the membrane vesicle by air exposure. If channel activity was observed, the patch was then exposed to a bath solution containing a higher Ca^{2+} concetration to examine Ca^{2+} dependency of the channel. Since channel activity declined rapidly upon prolonged exposure to $Ca^{2+} > 10^{-6}$ M (Saimi & Martinac, 1989), the patch was quickly moved back to the low Ca^{2+} solution (usually 10^{-8} M) to prevent further inactivation. The experimental chamber was constructed so as to facilitate rapid perfusion, as described in the previous paper (Saimi & Martinac, 1989).

Channels were exposed to 200 μg/ml proteases in the presence of 10^{-8} M to 10^{-5} M Ca^{2+}. The duration of each protease treatment (15 min for trypsin and pronase; 8–10 min for thermolysin) was decided after preliminary tests and was approximately 5 min longer than the time after an increase of channel activity appeared. Trypsin (type XII from bovine pancreas), trypsin inhibitor (type I-S from soybean) and thermolysin (protease type X, from *Bacillus thermoproteolyticus*) were purchased from Sigma (St. Louis, MO), and pronase (from *Streptomyces griseus*) from Calbiochem (San Diego, CA). The enzymes were dissolved and stored at 20 mg/ml in 200 μl aliquots at −20°C. The final dilution to 200 μg/ml was made just before actual use. Trypsin inhibitor and trypsin were mixed together before introducing them into experimental chamber.

Solutions

All the control bath solutions consisted of 100 mM K^+, 10^{-8} to 10^{-4} M free Ca^{2+} (buffered with 1 mM EGTA), 5 mM HEPES, 0.01 mM EDTA with 20 mM Cl^- and approximately 80 mM L-glutamic acid. The pH was adjusted to 7.0 with L-glutamic acid. EGTA was omitted from solutions containing 10^{-4} M Ca^{2+}.

Recordings and Data Processing

The methods used for recording and data analyses were similar to those described by Saimi and Martinac (1989). Since the number of channels and the channel activity varied from patch to patch, channel activity is expressed as normalized *NPo*. *NPo* was first calculated as the integral of channel current over a time period (13 sec), divided by the amplitude of single-channel current. Each *NPo* point for Figs. 3 and 4 was then normalized to an averaged *NPo* of the untreated channel at three negative voltages (−70, −50 and −30 mV in 10^{-4} M Ca^{2+}) where maximal channel activation occurs. There are relatively large scatters within this voltage range (*see* Fig. 7 of Saimi & Martinac, 1989), therefore no one voltage could be used as a reference level and *NPo* averaging was necessary. Significance of differences in the results was determined with *t* test at $P < 0.05$.

Table.

	Single-channel conductance (pS)	*n*
Before treatment	68 ± 5.8	10
Thermolysin-modified channels	68 ± 4.3	3
Trypsin-modified channels	73 ± 3.8	4
Pronase-modified channels	73 ± 3.8	3

Results

After giga-seal formation, we tested for the presence of hyperpolarization- and Ca^{2+}-dependent K channels in excised patches, as described in Materials and Methods, before proceeding with enzyme digestion. Figure 2 (left column) shows a typical current recording from patches containing a few active channels before protease treatment. Those channels had a conductance of about 70 pS (Table 1, "Before treatment"). They were activated by Ca^{2+} and were more likely to open upon hyperpolarization than upon depolarization. The unit conductance and Ca^{2+} and hyperpolarization dependence of these channels establish them as the channels of interest (*see* the companion paper by Saimi & Martinac, 1989).

Effects of Proteases

After identifying channels of interest, we then exposed membrane patches to proteases. Figure 1*A* (arrow) shows an abrupt change in the channel activity in the presence of thermolysin. There was a 2–3 minute lag before the sudden activation in thermolysin (six cases), but there was little change in channel activity thereafter over at least 15 min. Similar sudden changes were observed with the other proteases tested. After 8–15 min incubation, the chamber was perfused with a protease-free solution and channel activity examined at 10^{-8}, 10^{-6} or 10^{-4} M Ca^{2+} and at voltages between −70 and +70 mV. The channels after proteolytic modification could be blocked partially with 20 mM TEA (Fig. 1*C*, *D*). This blockage was comparable to that of the undigested K channels (*see* Fig. 8 of Saimi & Martinac, 1989), suggesting that the proteolysis occurred to the channels of interest. In all cases, protease treatment did not affect the single-channel conductance (Table).

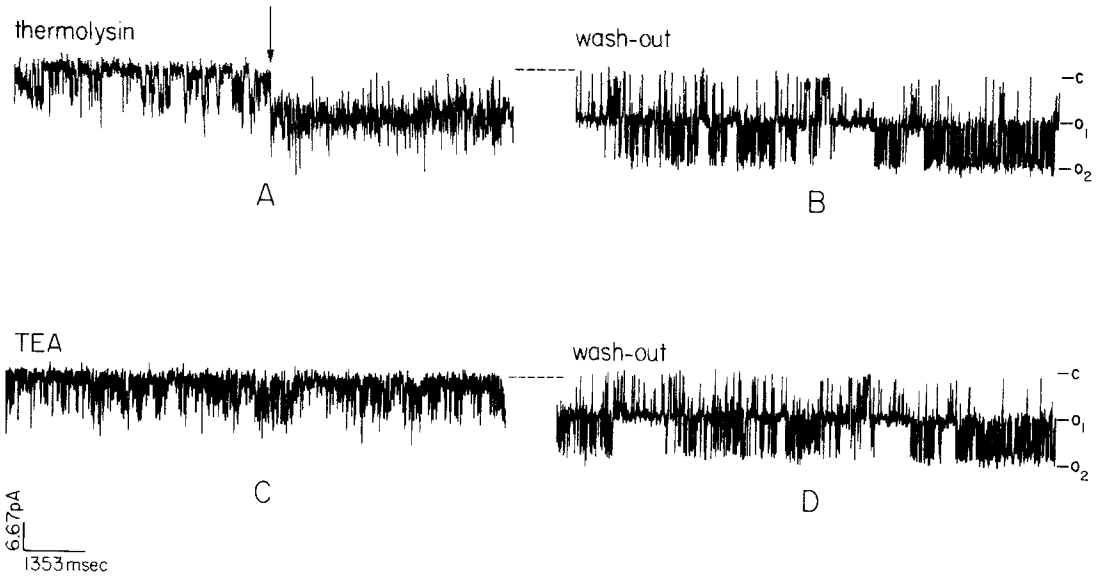

Fig. 1. Effect of 200 μg/ml thermolysin on channel activity in the presence of 10^{-5} M Ca^{2-}. (*A*) A recording in the presence of thermolysin in the bath. The patch was exposed to thermolysin 2 min prior to the trace shown here. The arrow indicates a rapid transition from the normal to the activated state of channel behavior. (*B*) A recording after 13 min of thermolysin treatment in the absence of thermolysin in the bath. The patch was then exposed to 20 mM TEA (*C*) to show blockage of the channels, compared to that after wash-out (*D*)

Trypsin

Trypsin treatment increased channel activity (Fig. 2*A*); the *NPo* of the channel increased by up to 20-fold in 10^{-6} M Ca^{2+} (*see* Figs. 3 and 4). The trypsin-treated channels were voltage dependent (*see* Fig. 3), but showed little difference in the normalized *NPo* at Ca^{2+} concentrations over four orders of magnitude (*see* Fig. 4), indicating a removal of the channel's Ca^{2+} dependence. Similar effects of trypsin on channel activity were observed when the enzyme was applied to the patch at a lower Ca^{2+} concentration (10^{-8} M).

As a control for possible nonenzymatic effects of trypsin, we exposed the patch first to a mixture of 200 μg/ml trypsin and 100 μg/ml trypsin inhibitor. The latter is described to be sufficient to inhibit the former (1 mg of inhibitor inhibits 1–3 mg of trypsin; *see* Sigma catalog). This exposure had little effect on the activity of the channels (Fig. 3). These patches were then subjected to trypsin digestion, which resulted in a similar increase of channel activity as described above.

Pronase

Like trypsin, pronase treatment increased channel activity (Fig. 2*B*), and the channel was still more active upon hyperpolarization than upon depolarization (Fig. 3). After the treatment, the channel was no longer Ca^{2+} dependent (Fig. 4). When pronase was applied at a lower Ca^{2+} concentration (10^{-8} M), we had to extend treatment time up to 25–30 min to produce effects similar to those described above.

Thermolysin

As with other proteolytic digestions, thermolysin treatment drastically increased the channel activity (Figs. 2*C*, 3 and 4). In line with pronase and trypsin, thermolysin removes Ca^{2+} dependence of activation at Ca^{2+} concentrations over four orders of magnitude (Fig. 4). Unlike the other treatments, however, thermolysin-treated channels also lost their voltage dependence (Fig. 3).

Discussion

We have described the effects of trypsin, pronase and thermolysin on the activity of a Ca^{2+}- and hyperpolarization-dependent K channel from *Paramecium*. In all cases application of proteases to the cytoplasmic face of the channel caused an irreversible increase in channel activity without affecting unit conductance. Trypsin and pronase similarly removed the Ca^{2+} dependence of channel activation, while thermolysin additionally removed the channel's voltage dependence. The channels after pro-

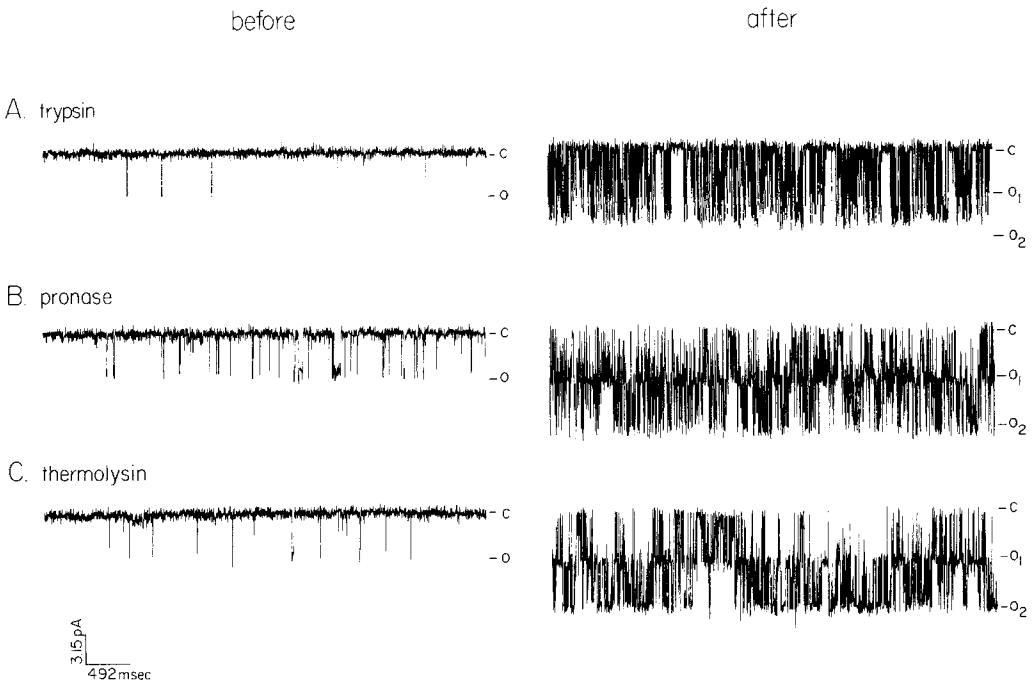

Fig. 2. Effects of three proteases on the *Paramecium* Ca^{2+}-dependent K channel. Membrane patches were first bathed in the 100 mM K solution, exposed to a protease described below for 8–15 min, and then brought into the 100 mM K solution without protease (*see* Materials and Methods for details). The channel activity was recorded at −50 mV in the presence of 10^{-6} M Ca^{2+} before and after protease treatments. (*A*) Trypsin effects. Left: before trypsin treatment; right: after trypsin treatment. (*B*) Pronase effects. Left: before; right: after. (*C*) Thermolysin effects. Left: before; right: after

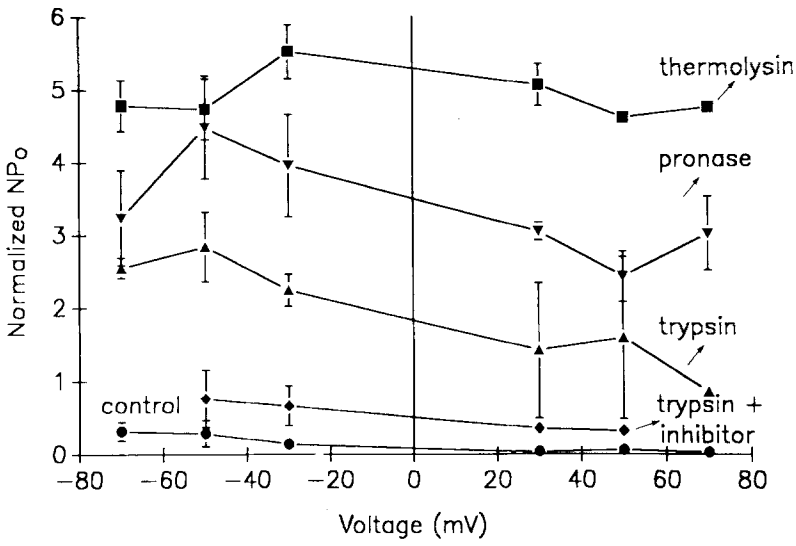

Fig. 3. Channel activity at 10^{-6} M Ca^{2+} expressed as normalized *NPo* before and after proteolytic modifications plotted against voltage imposed on the membrane patch (n = 3 or 4; mean ± SD). *NPo*'s were normalized to the average *NPo* values of the untreated channels in 10^{-4} M Ca^{2+} upon hyperpolarization (*see* Materials and Methods for details). A value of 1 for normalized *NPo* means a channel activity that is as high as that of untreated channels at −30 to −70 mV in the presence of 10^{-4} M Ca^{2+}. After pronase or trypsin treatment, the normalized *NPo* at all negative voltages was still significantly higher than at all positive voltages ($P < 0.05$). However, voltage sensitivity was lost by thermolysin treatment

teolytic modification retained their sensitivity to 20 mM TEA. These effects are presumably due to proteolytic digestion of the channel or its neighboring structure, since the effects of trypsin were suppressed by trypsin inhibitor.

Trypsin is the most specific of all proteolytic enzymes tested. It cleaves preferentially at lysyl and arginyl residues. Pronase is a mixture of at least seven proteolytic enzymes and is relatively unselective. However, one of the pronase components is alkaline proteinase *b*, a very specific endopeptidase that, like trypsin, cleaves lysine and arginine groups, and has been shown to remove an inactivation in sodium and voltage-sensitive potassium

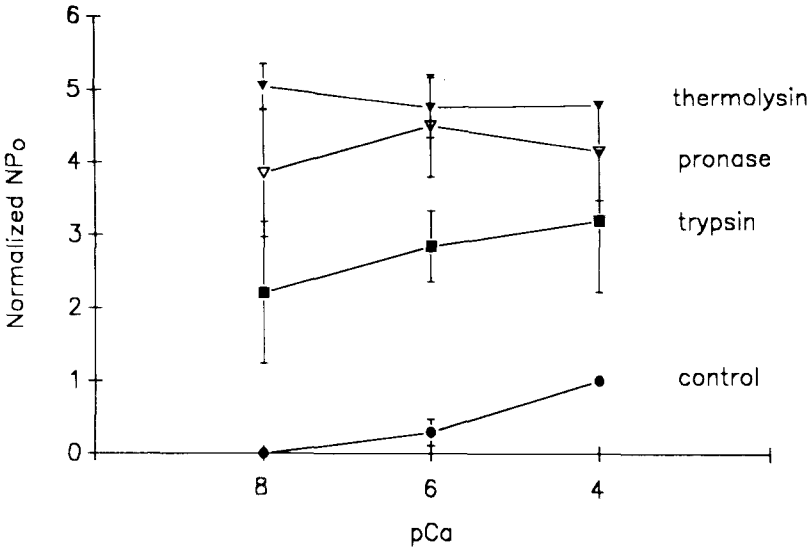

Fig. 4. Normalized *NPo* before and after proteolytic modifications plotted against Ca^{2+} concentration (n = 3 or 4; mean ± SD). The test membrane voltage was −50 mV. In all cases, channel activity is significantly ($P < 0.05$) higher after proteolysis than before; thus, Ca^{2+} sensitivity is removed

channels (Rojas & Rudy, 1976; Miller & Rosenberg, 1979). Our results on the Ca^{2+}- and hyperpolarization-activated channel from *Paramecium* have shown that both trypsin and pronase remove the Ca^{2+} dependence of channel gating within the concentration range of 10^{-8} and 10^{-4} M Ca^{2+} (Fig. 4). The channel, however, retains its voltage sensitivity (Fig. 3). While the effects of trypsin are Ca^{2+} independent, the duration of pronase treatment at low Ca^{2+} concentration (10^{-8} M) has to be extended to produce similar effects to those in the presence of 10^{-6} M Ca^{2+}. This suggests either that pronase digestion is Ca^{2+} dependent, and/or that the channel has to be in a certain state (presumably open) to be susceptible to digestion.

Thermolysin is an endopeptidase that hydrolyses amino groups of hydrophobic nonpolar amino acid residues including isoleucine, leucine, valine, phenylalanine, alanine and methionine. However, its action is not strictly limited to these hydrophobic amino acids residues. Of the three proteases tested here, thermolysin is most effective in removing both Ca^{2+} (Fig. 4) and voltage (Fig. 3) sensitivities from the *Paramecium* K channel. It should be emphasized that the thermolysin effects were very reproducible, and that the normalized *NPo* was consistently higher than after trypsin or pronase digestion. The fact that thermolysin removes both Ca^{2+} and voltage sensitivities of channel gating, while pronase or trypsin removes only the Ca^{2+} sensitivity, suggests that those two gating mechanisms may be conferred on the channel separately. Since thermolysin preferentially hydrolyses hydrophobic nonpolar amino acids, this may indicate that the voltage sensing is a property of a hydrophobic channel domain. The amphiphylic domain known as S4 region in Na (Noda et al., 1986) and K channel (Tempel et al., 1987), which comprises hydrophobic amino acids residues, may function as a voltage sensor. The Ca^{2+}-binding site, however, is most likely part of hydrophylic domain of the channel, therefore being attacked by proteases which cleave hydrophylic peptide bonds.

It has been argued that the large conductance BK channels may have Ca^{2+}-binding sites within the voltage drop across the membrane (Moczydlowski & Latorre, 1983; Latorre, 1986), which is reflected in the depolarization dependence of the BK channel activation. However, since the *Paramecium* K channel and SK channels in other organisms exhibit a weaker or an opposite voltage dependence from the BK channels (Blatz & Magleby, 1987), it seems that the Ca^{2+}-binding site for the *Paramecium* K channel and, perhaps, for the SK channels is differently located in the membrane. We suggest that the Ca^{2+}-binding site of the *Paramecium* K channel is on the cytoplasmic side, well outside of the voltage drop across the membrane.

Cleavage of peptide bonds on the BK channels have been shown in the rat skeletal muscle cells (Pallotta, 1985) and the thick ascending limb cells (Cornejo et al., 1987). In the both cases NBA was used. After NBA treatment, BK channels lose the Ca^{2+} dependence and are inactivated (Pallota, 1985), or the Ca^{2+} sensitivity becomes low, so that a much higher Ca^{2+} concentration is necessary for the channel activation (Cornejo et al., 1987). Our preliminary results suggest that NBA, like proteases, causes an irreversible activation of the *Paramecium* K channel (*unpublished*). This observation, together with other properties of the channel, suggests that the structure of the hyperpolarization-

and Ca^{2+}-activated *Paramecium* K channel is very different from that of the BK channels.

A priori, one could imagine that a Ca^{2+} binding could cause a configuration change in the channel protein thereby, generating the K^+ pathway. Alternatively, this pathway may already exist but is obstructed or inhibited by a protein domain in the absence of Ca^{2+}. Ca^{2+} binding removes this obstruction or inhibition and reveals the existing ion pathway. A similar activation model involving removal of a gate by Ca^{2+} binding was proposed for BK channels (Methfessel & Boheim, 1982). We suggest that under normal conditions, inhibition of *Paramecium* K channels is relieved by Ca^{2+} binding to the inhibitory domain, and that this inhibitory domain may be removed during proteolytic digestion.

Many Ca^{2+}/calmodulin-dependent enzymes can be irreversibly activated with trypsin or other proteases (Niggli, Adunyah & Carafoli,1981; Tucker, Robinson & Stellwagon, 1981; Walsh et al., 1982; Klee, Krinks & Manalan, 1983). Limited proteolysis of calcineurin irreversibly removes the Ca^{2+}-binding domain and the protease-induced activity of the enzyme is much higher than upon the normal activation by Ca^{2+} (Klee et al., 1983). Similarly, a limited digestion of Ca^{2+}/calmodulin-dependent myosin light chain kinase of smooth muscle yields a very active and Ca^{2+}-independent form of the enzyme (Walsh et al., 1982). There is clearly an analogy between the effects of proteases on these enzymes and on the Ca^{2+} mediation of the channel of *Paramecium*. It is possible that Ca^{2+} mediation of the channel might be through its binding to calmodulin or a calmodulin-like element of the channel. Further studies are required to clarify this possibility.

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