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Effects of Proteolytic Enzymes on Ionic Conductances of Squid Axon Membranes

Carlos Sevcik* and Toshio Narahashi

Department of Physiology and Pharmacology, Duke University Medical Center
Durham, North Carolina 27710

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Summary. The effects of proteolytic enzymes on ionic conductances of squid axon membranes have been studied by means of the voltage clamp technique. When perfused internally α-chymotrypsin (1 mg/ml) increased and prolonged the depolarizing after-potential. Sodium inactivation was partially inhibited causing a prolonged sodium current, and peak sodium and steady-state potassium currents were suppressed. The time for sodium current to reach its peak was not affected. Leakage conductance increased later. On the other hand, carboxypeptidases A and B, both at 1 mg/ml, suppressed the sodium and potassium conductance increases with little or no change in sodium inactivation. The mechanism that controls sodium inactivation appears to be associated with the structure of membrane proteins which is modified by α-chymotrypsin but not by carboxypeptidases and is located in a position accessible to α-chymotrypsin only from inside the membrane.

Previous studies have demonstrated that electrical activity of the giant fiber of the squid is modified when perfused internally with solutions containing proteolytic enzymes (Tasaki & Takenaka, 1964; Rojas & Atwater, 1967; Armstrong, Bezanilla & Rojas, 1973). These enzymes are known to disrupt specific bonds between amino acids in proteins and polypeptides. In order to gain more insight into the role of proteins in the molecular processes underlying nerve excitation, we have studied the effects of α-chymotrypsin, carboxypeptidase A, and carboxypeptidase B on ionic conductances of squid axon membranes.

A preliminary account of this paper has been reported (Sevcik & Narahashi, 1973).

* *Present address:* Centro de Biofisica y Bioquimica, Instituto Venezolano de Investigaciones Cientificas (IVIC), Apartado 1827, Caracas 101, Venezuela.

Materials and Methods

Material and Electrical Measurements

Giant axons from the hindmost stellate nerve of the squid *Loligo pealei* were internally perfused by the method originally developed by Baker, Hodgkin and Shaw (1961), and modified by Narahashi and Anderson (1967). The axon was mounted horizontally in a nerve chamber. Membrane currents were measured by axial wire voltage clamp technique (Wang, Narahashi & Scuka, 1972). Internal electrode consisted of a glass capillary and a platinum wire attached piggy-back. The glass capillary was 75 μ in outer diameter, contained a floating 25 μ platinum wire to reduce high frequency impedance, and was filled with 0.6 M KCl solution. This was used to measure internal potential. The piggy-back platinum wire was 75 μ in diameter, and was insulated with wax except for the terminal portion (13 mm) where platinum black was coated for current stimulation. External reference electrode was a glass capillary, 200 μ in diameter, and contained artificial seawater. External current electrodes consisted of three platinum black foils, 4 mm wide each, placed on one side of the axon. The central plate was connected with the summing point of an operational amplifier to measure currents passing through the nerve membrane, and the two side plates were directly grounded to serve as the guard electrodes.

Solutions

The axons were immersed in artificial seawater of the following composition (mM): Na^+ 449, K^+ 10, Ca^{++} 50, Cl^- 572, Tris (tris(hydroxymethyl)aminomethane) 30, at final pH 8.0. The high calcium concentration was used to prevent repetitive firing in internally perfused axons. Standard internal solution contained monoglutamate$^-$ 320, F^- 50, HPO_4^- 15, Na^+ 50, K^+ 350 mM at final pH 7.3. All experiments with carboxypeptidases were carried out at room temperature (22 °C), and those with α-chymotrypsin at 10 °C.

Enzymes

Enzyme was added to the internal solution at a concentration of 1 mg/ml immediately before each experiment. α-Chymotrypsin, purchased from Worthington Biochemical Corp., N.J., was an activation product of 3× chrystallized zymogen, and a dialyzed, salt-free, lyophylized powder. Carboxypeptidase A was treated with diisopropylfluorophosphate (DFP) to eliminate tryptic and chymotryptic activities, recystallized after removal of the DFP, and shipped as an aqueous suspension with toluene added as a preservative (Worthington). Carboxypeptidase B was treated with DFP (Worthington), and contained negligible amounts of tryptic and chymotryptic activities. It was shipped as a frozen solution in 0.1 M NaCl.

Statistics

Student's *t*-test was used to determine the significance of difference between means unless otherwise stated. A confidence level of 5% or less ($p < 0.05$) was considered to be significant. Results are expressed as the mean ± standard error of the mean.

Results

α-Chymotrypsin

Fig. 1 illustrates the effect of α-chymotrypsin on resting and action potentials. After an initial transient depolarization, the membrane was hyperpolarized slightly and the spike was followed by a depolarizing afterpotential. Later, the membrane was depolarized slowly, the action potential was decreased in amplitude progressively, and finally the membrane was rendered inexcitable. Unlike the observation by Tasaki and Takenaka (1964), no repetitive firing occurred following internal perfusion with α-chymotrypsin. The absence of repetitive firing is presumably due to the high calcium concentration in our artificial seawater.

Fig. 2 illustrates the membrane current associated with a step depolarization from the holding membrane potential of −60 mV to −10 mV. After enzyme was added, both peak sodium current and steady-state current were decreased. The steady-state current was partially restored when 300 nM tetrodotoxin (TTX) was applied externally to the enzyme-treated axon. Since the membrane current recorded after application of α-chymotrypsin and TTX contains no sodium current, the subtraction of this membrane current (Fig. 2, right tracing) from the membrane current in α-chymotrypsin (Fig. 2, middle tracing) represents the sodium current in α-chymotrypsin. It is clear that a prolonged sodium current is flowing 7 msec after the start of the depolarizing step.

Current-voltage curves are illustrated in Fig. 3. Twenty minutes after internal perfusion of α-chymotrypsin, the peak sodium current (open circles) was reduced in amplitude (open triangles), while the steady-state current (filled circles) was shifted along the potential axis in the direction

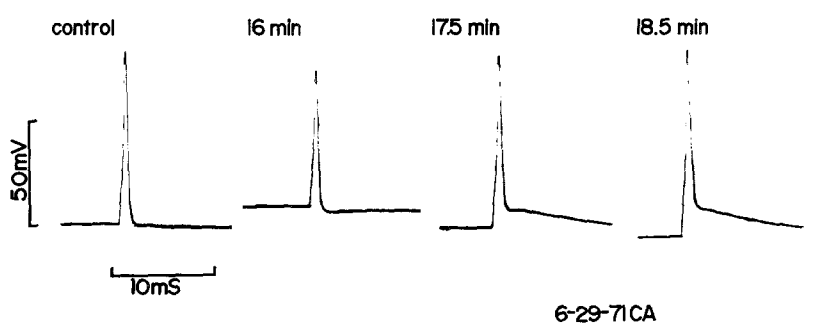

Fig. 1. Effects of internal perfusion on 1 mg/ml α-chymotrypsin on resting and action potentials. The resting potential prior to enzyme application is −54 mV

Fig. 2. Membrane currents associated with step depolarization from the holding membrane potential of −60 mV to −10 mV before and after internal application of 1 mg/ml α-chymotrypsin (α-ChT), and after external application of 300 nM tetrodotoxin (TTX). Note the different current calibration for the α-ChT record

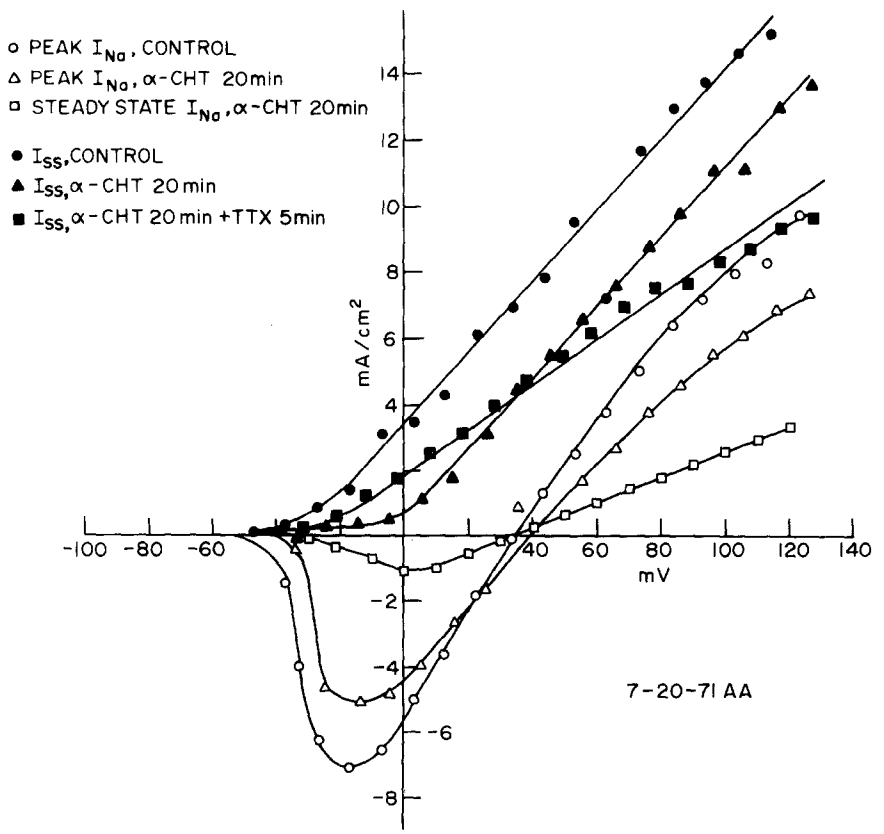

Fig. 3. Current-voltage relations for peak sodium current (I_{Na}) and for steady-state current (I_{ss}) before and after internal perfusion of 1 mg/ml α-chymotrypsin (α-ChT) and after external application of 300 nM tetrodotoxin (TTX). Open squares represent steady-state sodium current obtained by subtraction of the steady-state current in α-ChT plus TTX from that in α-ChT

of depolarization (filled triangles). The steady-state current observed after external application of 300 nM TTX to the axon internally perfused with α-chymotrypsin is represented by filled squares. The steady-state current

was increased by treatment with TTX at the membrane potentials more negative than the reversal potential for peak sodium current, and was decreased otherwise. Open squares represent the difference between the steady-state currents with and without TTX after internal perfusion with α-chymotrypsin. This residual current has the same reversal potential as the peak sodium current. Therefore, it is carried by sodium ions.

When the membrane is suddenly repolarized after a brief period of depolarization, a rapid surge of capacitative current flows inward. This capacitative current is immediately followed by an ionic tail current (Hodgkin & Huxley, 1952). The time course of sodium conductance change during a step depolarization can be obtained by plotting the peak amplitudes of the tail sodium currents.

The membrane was depolarized from the holding level of −60 mV to 0 mV, and was repolarized to −60 mV at various moments. The capacitative current observed at the beginning of the depolarizing step was subtracted from the tail currents associated with step repolarizations, and the amplitudes of the corrected tail currents are plotted against time in Fig. 4 (open circles). Following internal perfusion of α-chymotrypsin,

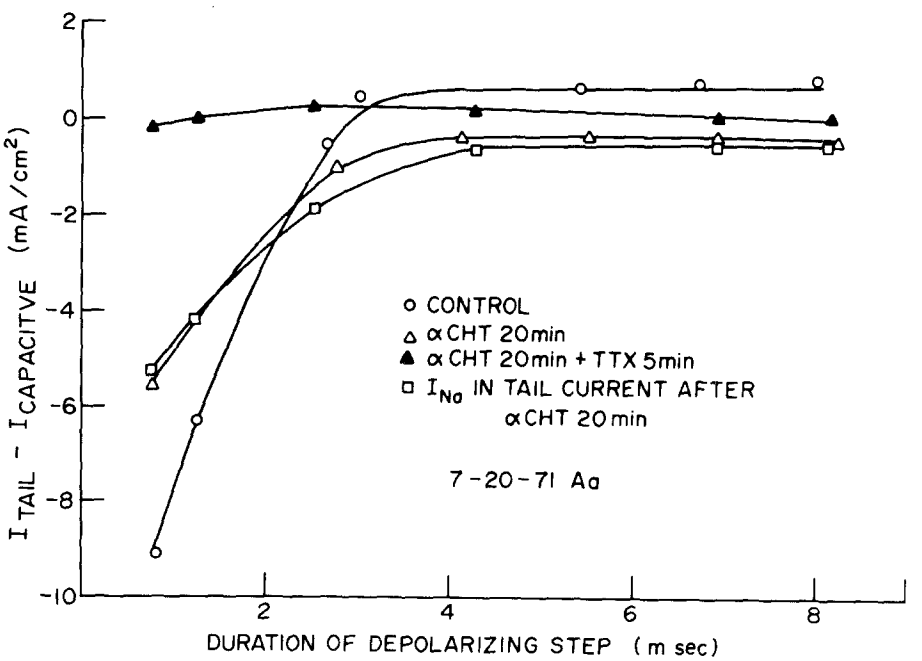

Fig. 4. Sodium "tail" currents during the course of a step depolarization from the holding membrane potential of −60 mV to −10 mV before and after internal perfusion of 1 mg/ml α-chymotrypsin (α-ChT) and external application of 300 nM tetrodotoxin (TTX). *See text* for further explanation

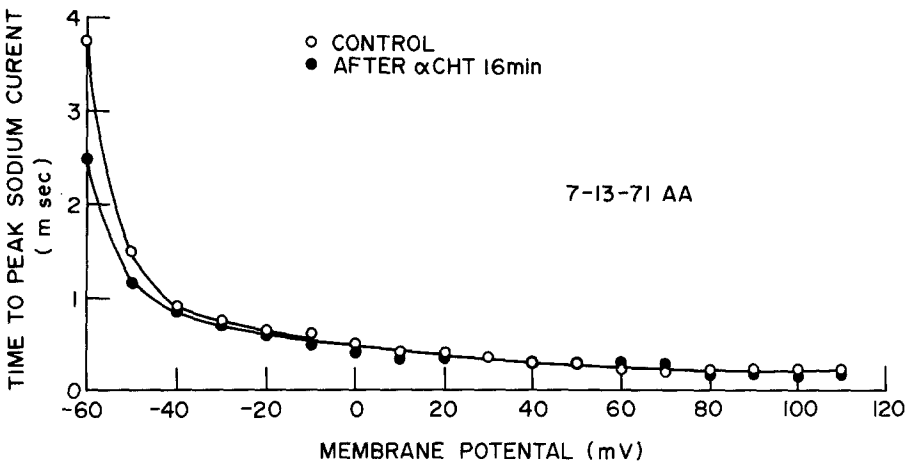

Fig. 5. Time for sodium current to reach its peak before and after 16 min of internal perfusion of 1 mg/ml α-chymotrypsin (α-ChT)

the tail current was decreased during a short depolarizing step of about 2 msec, but was increased with an increase in the duration of pulse (open triangles). External application of 300 nM TTX almost completely abolished the tail current (filled triangles). The difference between the tail currents before and after application of TTX is plotted as open squares, and represents the time course of tail sodium current during a depolarizing step. Steady-state sodium current is still flowing 8 msec after the onset of step depolarization.

The time required for the sodium current to reach its peak gives some information on the activation kinetics of the sodium channel. The time to peak is plotted as a function of the membrane potential in Fig. 5. Internal perfusion of α-chymotrypsin had little or no effect on this parameter. The paired student's *t*-test indicates that the difference between the control and α-chymotrypsin is not statistically significant ($p > 0.05$).

Carboxypeptidase A

The time course of the effects of carboxypeptidase A at 10 °C is very slow. In one experiment, the axon was perfused internally with carboxypeptidase A for more than one hour with little or no change in the amplitude or the time course of membrane currents. At room temperature, carboxypeptidase A produced a slight hyperpolarization of about 5 mV. It usually appeared about 15 min after the start of perfusion with enzyme and lasted up to 3 hr. Hyperpolarization terminated and depolarization began when leakage conductance increased.

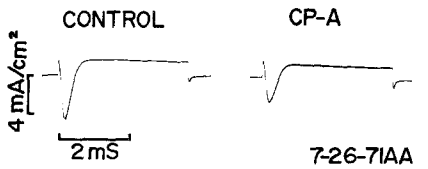

Fig. 6. Membrane currents associated with step depolarization from the holding membrane potential of −80 mV to −10 mV before and after internal perfusion of 1 mg/ml carboxypeptidase A (CP-A)

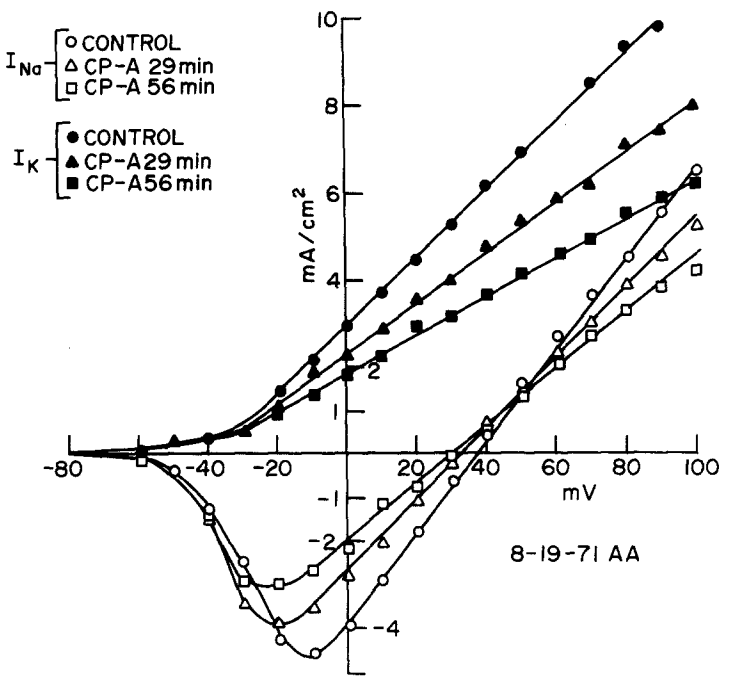

Fig. 7. Current-voltage relations for peak sodium current (I_{Na}) and for steady-state potassium current (I_K) before and after internal perfusion of 1 mg/ml carboxypeptidase A (CP-A). Holding membrane potential −80 mV

The membrane current associated with a step depolarization from the holding membrane potential of −80 mV to −10 mV are illustrated in Fig. 6. After internal perfusion with carboxypeptidase A, both peak and steady-state currents were reduced in amplitude. However, no appreciable change in the time course of membrane current occurred in any of the seven axons tested.

Fig. 7 represents current-voltage curves from another axon internally perfused with carboxypeptidase A. Both peak sodium and steady-state potassium currents continued to decrease as the perfusion with carboxy-

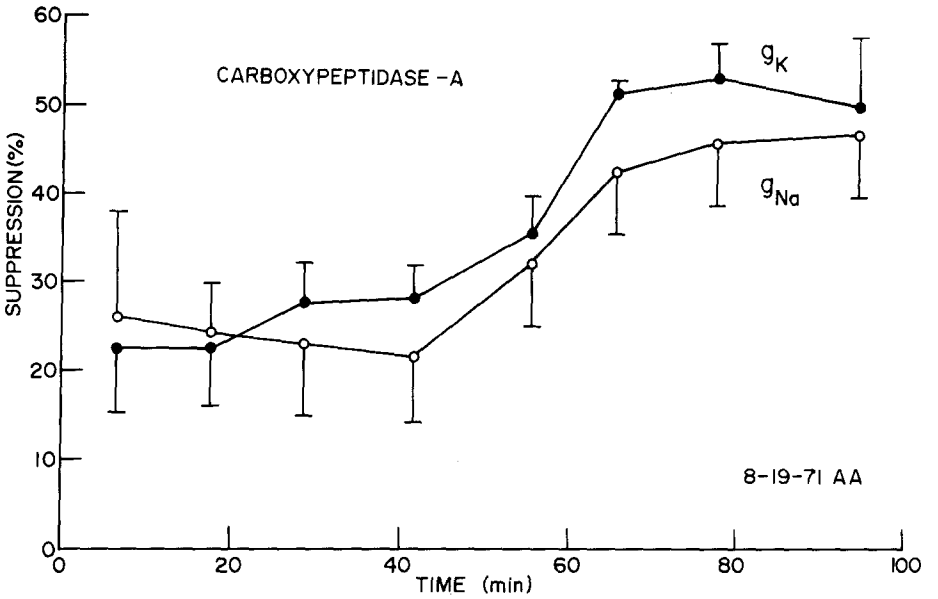

Fig. 8. Time course of suppression of sodium (g_{Na}) and potassium (g_K) conductances during a prolonged internal perfusion of 1 mg/ml carboxypeptidase A in a squid axon. Circles represent the mean percentage of suppression and bars standard errors of means. The suppression produced by the enzyme was independent of the membrane potential and each mean was calculated from 17 measurements at the membrane potentials ranging from -40 mV to 120 mV at 10-mV steps

peptidase A was maintained. The membrane potential where the sodium current reaches a maximum value was slightly shifted in the hyperpolarizing direction. Such a shift occurred in four out of seven axons tested, being a statistically significant fraction ($57.1 \pm 18.7\%$, $p < 0.01$) as calculated by the method given by Fleiss (1973).

The chord conductances were calculated for the sodium and potassium components, and the reductions produced by the enzyme are plotted as a function of the time after application of carboxypeptidase A in Fig. 8. It is clear that carboxypeptidase A reduced both conductances to approximately the same extent during a prolonged perfusion.

In contrast to α-chymotrypsin, carboxypeptidase A did not modify the time course of the sodium current. Leakage conductance started increasing long after sodium and potassium conductances decreased. It was not possible to determine whether the leakage conductance increase was produced by enzyme action or by deterioration of preparation.

Carboxypeptidase B

As in the case of carboxypeptidase A, both sodium and potassium currents were decreased by internal perfusion with carboxypeptidase B. The effects were augmented as the perfusion was continued. No change in the time course of membrane currents was observed with carboxypeptidase B.

In four experiments, the effects of carboxypeptidase B were more marked on sodium conductance than on potassium conductance. During the course of a prolonged perfusion with carboxypeptidase B, the sodium conductance was suppressed to a greater extent than the potassium conductance, although the difference between them at any particular time was either small or statistically insignificant.

Discussion

When perfused internally, α-chymotrypsin suppresses both peak sodium and steady-state potassium currents and prolongs sodium inactivation. More drastic inhibition of the sodium inactivation has been observed with the squid axon internally perfused with pronase (Armstrong *et al.*, 1973). However, the action of pronase, like that of α-chymotrypsin, is not totally specific for the sodium inactivation, inhibiting the peak and steady-state currents to some extent (Yeh & Narahashi, *unpublished observations*). It should be noted that both α-chymotrypsin and pronase are endopeptidases.

Despite the potent action of α-chymotrypsin from the internal membrane surface, it has no effect on electrical excitability of squid axons when applied to the external membrane surface (Tasaki & Takenaka, 1964). Thus the mechanism that controls sodium inactivation appears to be associated with the structure of membrane proteins which is modified by α-chymotrypsin and is located in a position accessible to the enzyme only from inside the membrane.

Carboxypeptidases A and B are both exopeptidases, and inhibit both peak sodium and steady-state potassium currents without affecting sodium inactivation. They specifically remove terminal carboxylamino acids. In view of their blocking actions on both conductances, it is suggested that acyl-amino acids, located on or near the internal membrane surface, play important roles in controlling the sodium and potassium gating mecha-

nisms. Due to the high specificity of carboxypeptidase A for phenylalanine and tyrosine (Narita, 1970; Pétra, 1970), they are probably terminal amino acids.

The blocking action of carboxypeptidase B on both conductances gives evidence in favor of the existence of terminal carboxylamino acids such as lysine, arginine and serine at the end of peptidic chains (Folk, 1970; Narita, 1970). In view of the fact that both carboxypeptidases are effective on the membrane currents, it is likely that there is a protein with more than one terminal carboxylamino acid or that several protein subunits are involved in the structure of the gating mechanisms of ionic channels.

The pK_a for the carboxyl groups of all amino acids commonly occurring in proteins lies between 1.65 and 3.86 (Lehninger, 1970). Thus at the pH of 7.3 of the internal perfusate, they will be ionized and bear part of the fixed charges at the inner phase of the membrane. It seems likely that the removal of such charges may be one of the mechanisms by which carboxypeptidases modify ionic permeability.

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