Biochimica et Biophysica Acta, 693 (1982) 188–194
Elsevier Biomedical Press

BBA 71409

REMOVAL OF Na^+ CHANNELS IN SQUID GIANT AXONS BY PERFUSION WITH TRYPSIN

EMILIO CARBONE

Istituto di Cibernetica e Biofisica del CNR, 16032, Camogli (Italy)

(Received July 19th, 1982)

Key words: Ion channel; Gating current; Voltage clamp; Trypsin; (Squid giant axon membrane)

The irreversible effects of the proteolytic enzyme trypsin on ionic and gating currents of voltage-clamped squid axon membranes have been studied. At physiological pH, internal perfusion of the fibre with trypsin was found to be very effective in removing Na^+ channels leaving the potassium system almost unaltered. At $T = 13°C$ the rates of channel-cleavage averaged $1/10$ min^{-1} for the Na^+ and $1/128$ min^{-1} for the K^+ channel, respectively. As estimated by the decrement of peak sodium conductance, the rate of loss of Na^+ channels correlates well with the rate of decrease of the total charge associated with the ON component of gating currents, indicating that trypsin probably interacts with an essential proteic portion of the channel whose removal might prevent both the displacement of gating charges and the subsequent opening of the channel. Intracellular pH remarkably influences the action of the enzyme. A plot of the pH-dependence of the rate of cleavage of Na^+ channels suggests the involvement of a positively charged group (either lysine or arginine) in the substrate region of the trypsin catalytic reaction.

Introduction

Opening and closing of ionic channels in nerve membranes are regulated by charged structures (gating particles) that move in response to changes of the membrane electric field [1–3]. Attempts have been made to elucidate their chemical nature, but despite the amount of data accumulated (for a recent review, see Refs. 4 and 5) the present picture of the activation gating machinery still remains obscure. More quantitative evidence has been obtained for the fast inactivation process (h-gate) of the Na^+ channel of squid axons. Briefly, the h-process was shown to be gated by a polypeptide sequence which in part is located near the inner side of the pore [6,7], and contains arginine and tyrosine residues [8–11]. I report here some new findings concerning the molecular groups that may be involved in the turning-on mechanism of the Na^+ channels.

Like other proteases [12], trypsin is known to affect the electrical excitability of squid giant axons only when applied intracellularly [13,14]. In crayfish axons internal application of the enzyme at low concentrations (0.02 mg/ml) produces a modification of the slow sodium inactivation [15], while in mollusc neurons external application of the enzyme for short periods of time inhibits the action of tetrodotoxin (TTX) on sodium currents [16,17]. The present work shows that at high concentrations (2 mg/ml) trypsin acts at the intracellular side of the axon membrane mainly by destroying Na^+ channels and their activation gates. The action is reduced below pH 7.2 and enhanced above, following a pH-dependence close to that found in vitro for the trypsin-catalysed hydrolysis of arginine or lysine derivatives, which are the classical substrates of the enzyme [18]. This suggests that positively charged groups at the inner side of the channel might be intimately related to

the functioning of the activation gate.

Some of the results presented here have also been reported in a preliminary communication [19].

Materials and Methods

Axon preparation, voltage-clamp, stimulating-recording system

All experiments were performed on internally perfused giant axons of the squid *Loligo vulgaris* (350–700 μm in diameter), available in Camogli. The axon chamber, the perfusion technique and the temperature controlling system were similar to those used in previous studies [11,20]. The geometry of the external current and guard electrodes was modified: the electrodes were platinized-silver cylinders with a diameter of 2.2 mm and a length of 4 and 7 mm, respectively.

Membrane currents were digitized by a 12 bit analog-to-digital converter (ADC) at intervals varying between 8 and 512 μs. Generation of voltage command pulses and storage of the sampled records were accomplished on-line by suitably programmed microprocessors [21]. Sampled data were transferred off-line to a DECLAB minicomputer (Digital Equipment Co., MA, U.S.A.) for further analysis (RSX-11 operating system).

The membrane series resistance, R_s, was measured using, in a current-clamp configuration, the same electronic set-up used for voltage clamping the fibre. When needed, 90% of R_s was compensated by means of a positive feedback-loop [22]. Membrane currents were corrected for capacitive transients by an analog circuit and recorded after filtering at 50 KHz with a cascade of two 4-pole low-pass RC filters (Mod. 3323, Krohn-Hite Co., MA, U.S.A.). Pulse procedures for measuring sodium and potassium currents were identical to those described elsewhere [11,20].

Gating currents were measured using the P/4 pulse procedure of Armstrong and Bezanilla [23]. The conditioning potential before test was −80 mV. The potential from which the four correcting pulses were delivered (V_n) was −128 mV. V_n values less negative than those usually employed (−170 mV) were used in order to allow better leakage compensation in the trypsin-treated fibres (having larger and more non-linear leakage currents than control fibres). Control runs at V_n = −160 mV in axons barely treated with the enzyme yielded similar results.

Solutions

The composition of internal and external solutions used is given in Table I. The enzyme was added to the internal solution at a concentration of 2 mg/ml just before each measurement. Particular care was taken to maintain a constant flow of solution inside the fibre during the experiment. Two types of trypsin from bovine pancreas (type

TABLE I

IONIC COMPOSITION OF THE SOLUTIONS IN mM

ASW, artificial sea water; TMA, tetramethylammonium (BDH, London).

External	Na^+	K^+	TMA	$Tris^+$	Ca^{2+}	Mg^{2+}	Cl^-	pH (±0.1)
ASW	435	10		20	10	40	555	8
1/4Na-SW	109	10	326	20	10	40	555	8
Tris-SW				525	10	40	494	7.6
Internal [a]	Na^+	K^+	Cs^+	F^-	H_2PO_4			pH (±0.1)
400 K		400		317	45			7.2
50 Na	50	135	215	317	45			7.2
350 Cs	27		350	350	15			7.2
200 Na	200			117	45			7.2

[a] Sucrose was added to maintain an osmolarity of 1070 mosmol.

III and XII, Sigma Chem. Co., St. Louis, MO) and one from pig pancreas (Miles Lab. Lim., Goodwood, South Africa) were employed without further purification. They yielded essentially similar results although the Miles product was found to be specific in removing Na^+ channels. Trypsin (Miles) had a specific activity of approx. 14000 BAEE (α-*N*-benzoyl-L-arginine ethyl ester) units per mg of material and an associated chymotrypsin activity of 7 units/mg. The product was a generous gift of Professor C. Romanzi (Istituto di Microbiologia, Genova Universitá di Genova).

To determine the pH-dependence of the enzyme activity, the concentration and quality of buffers employed were similar to those described elsewhere [20].

Results

Effects of Na^+ conductance and steady-state Na^+ inactivation

The selective action of trypsin on Na^+ currents is illustrated in Fig. 1. After perfusing the axon internally with trypsin (2 mg/ml) for 15 min, inward and outward Na^+ currents are irreversibly depressed, while 75% of the K^+ currents retain their kinetic properties. Progressive increases of leakage currents were normally observed during these measurements.

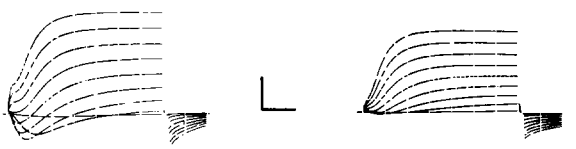

Fig. 1. Voltage-clamp records associated with step depolarizations from −90 mV, before and after 15 min of internal perfusion with 2 mg/ml of trypsin Miles. Test potentials were (mV): −50, −10, +10, +30, +50, +70, +90, +110, +130. Bars: 4 mA·cm^{-2}, 1 ms. Axon 1-JLO5O. In: 400 K; Out: artificial sea water (ASW). $T = 13°C$.

As shown in Fig. 2a, trypsin markedly reduces the peak amplitude of Na^+ conductance (g_1) and, to a minor degree, the residual conductance levels

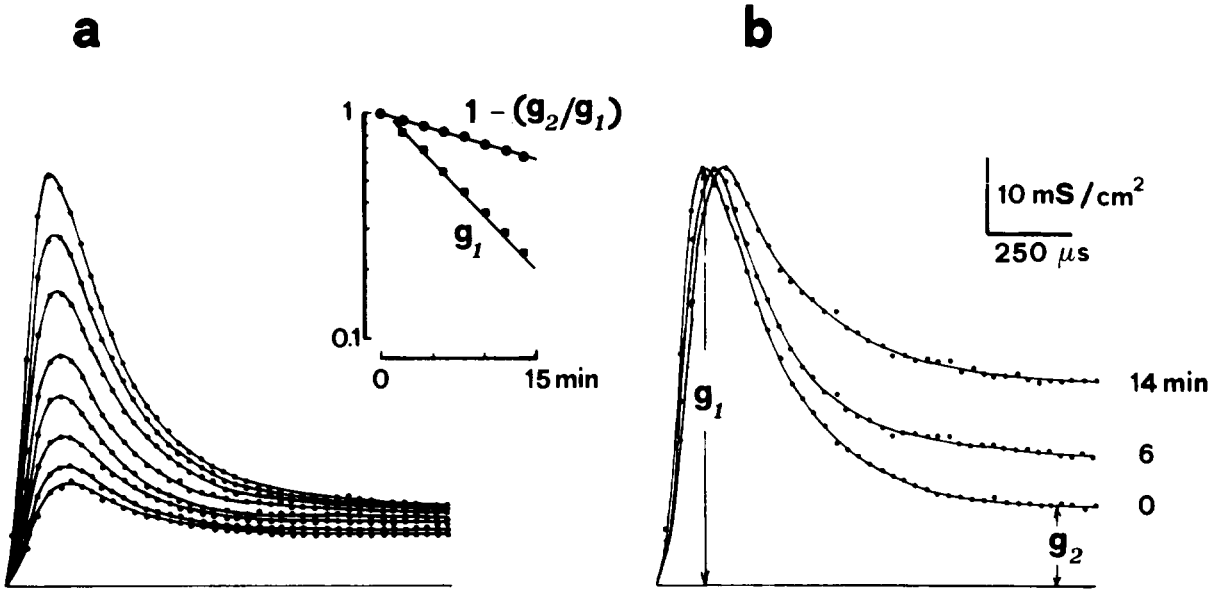

Fig. 2. (a) Computer records of Na^+ conductance g_{Na} associated with step depolarizations to +100 mV from −90 mV. Voltage pulses were delivered at 2-min intervals from the start of trypsin application (2 mg/ml). Axons were internally perfused with a solution containing 215 mM Cs^+ to block K^+ outward currents. Records are corrected for tetrodotoxin-insensitive currents. Solid lines through dots are drawn by eye. E_{Na} was +32 mV. Axon 2-JLO7O. In: 50 Na. Out: 1/4 Na-SW. $T = 13°C$. (b), Normalized g_{Na} records taken from part (a), before ($t = 0$) and after 6 or 14 min perfusion with trypsin. The slight increase of the time-to-peak caused by the enzyme could be due to a small shift toward depolarizing voltages of the activation time constant as found for g_{Na} (E) in Fig. 3a. Inset: Semilog plot of the time-course of g_1 and $1-(g_2/g_1)$; g_1 was determined at the peak of g_{Na} and g_2 at the end of the pulse as indicated. Straight lines through dots are best fittings with slope of 1/9 and 1/31 min^{-1}, respectively.

(g_2), producing only small changes of the activation and inactivation kinetics (Fig. 2b). Significantly, the decrease of g_1 and $1-(g_2/g_1)$ develops at different rates (see inset): 1/9 min^{-1} for g_1, and 1/31 min^{-1} for $1-(g_2/g_1)$, the latter being plotted in place of g_2 because it provides a more reasonable measure of the fraction of functioning channels normally inactivated (see p. 399 and Fig. 4 in Ref. 11).

As illustrated in Fig. 2b, the action of trypsin on fast Na^+ inactivation quite closely resembles that of pronase (a mixture of proteolytic enzymes) [6]. Thus, besides reducing the number of functioning channels, trypsin probably also destroys the fast Na^+ inactivation of a fraction of channels that are opened on step depolarizations. Two further observations support this view: First, the voltage-dependence of sodium conductance in fibres heavily treated with trypsin (g_3) (2 mg/ml for 30 min at $T = 13°C$) is more similar to that measured at the peak than that measured at the steady-state of untreated fibres (Fig. 3a). Similar findings have previously been reported for pronase (see p. 400 and Fig. 7 in Ref. 11). Second, like pronase and other inactivation-removing agents [7–10] trypsin irreversibly affects steady-state Na^+ inactivation ($1-h_\infty$) (Fig. 3b).

At 2 mg/ml trypsin does not influence the steady-state slow Na^+ inactivation (s_∞). An effect on slow Na^+ inactivation has been described for crayfish axons at a trypsin concentration of 0.02 mg/ml [15]. Since I did not study this much lower

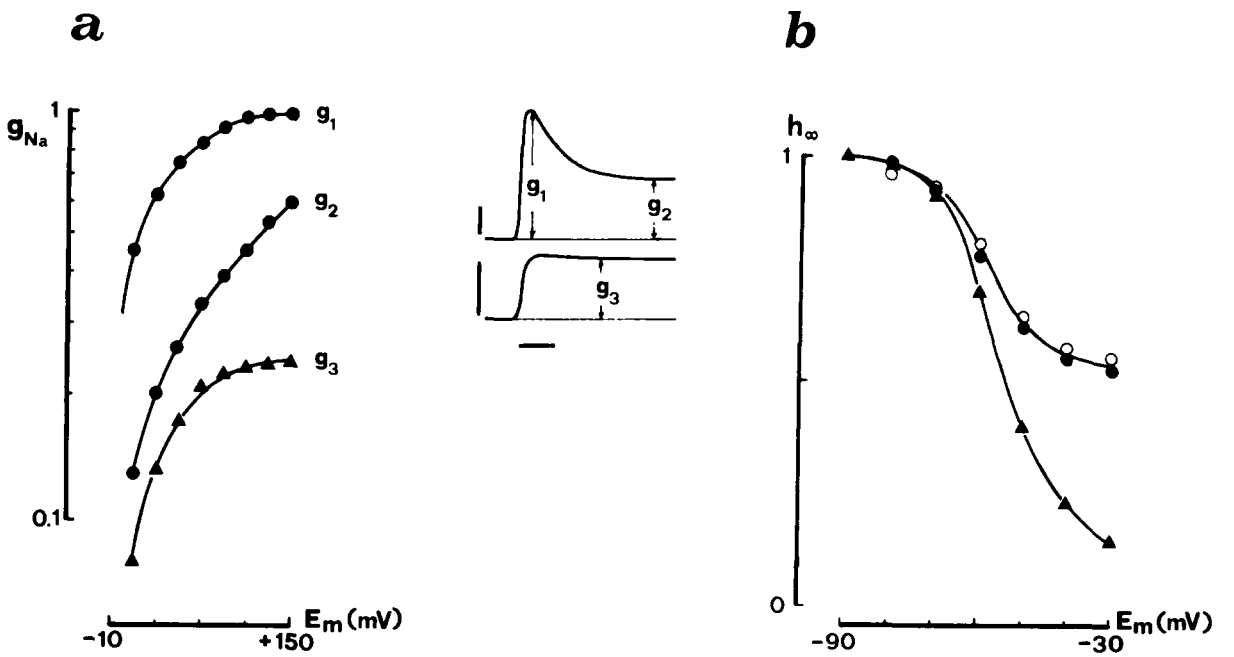

Fig. 3. (a) Normalized g_{Na} as a function of membrane potential, E_m, before (circles) and after 30 min perfusion with 2 mg/ml trypsin XII (Sigma Chem. Co.) (triangles). Solid lines through experimental points are drawn by eye. g_1, g_2 and g_3 were determined at the times indicated in the inset. Curve g_3 is the same as g_1 but shifted 8 mV to the right and scaled in amplitude to fit the triangles. Compared to Fig. 2a, the ratio g_2/g_1 at +100 mV appears to be larger (0.45 against 0.2 of Fig. 2a). This is typical of axons perfused with high levels of internal Na^+ (200 against 50 mM in Fig. 2a) (see Ref. 24). For this axon, as well as for two others perfused with 200 Na, the rate of decrement of g_1 was found to be approx. 1/10 min^{-1} for the first ten min, and 1/35 min^{-1} for the remaining twenty. This explains why g_3 at +100 mV is nearly 0.2 g_1 after 30 min of trypsin-treatment rather than 0.03 g_1, as expected if g_1 decreased at a rate of 1/9 min^{-1} as in Fig. 2a. Such different behaviour could be due to the presence of high internal Na^+, but this phenomenon was not further investigated. Inset: g_{Na} records taken before (above) and after (below) enzyme treatment. E_{Na} was −10 mV. Bars: 1 mA/cm², 0.5 ms. Axon 1-MA200. In: 200 Na. Out: 1/4 Na-SW. $T = 13°C$. (b) Voltage-dependence of h_∞, before (triangles) and after 14 min perfusion with trypsin XII (Sigma Chem. Co.) (circles). Open circles refer to test pulses to +80 mV, the filled ones to 0 mV. Conditioning prepulses of varying amplitude lasted 40 ms. Axon 2-Ma130. In: 50 Na. Out: 1/4 Na-SW.

concentration range, two possibilities are suggested: either the effects observed on crayfish axons at 0.02 mg/ml are completely masked by the much higher concentrations used in the present experiments, or the enzyme acts differently on different nerve preparations. In agreement with previous studies [14] no sizeable effects were found when trypsin was applied extracellularly at 2 mg/ml.

Action of trypsin on gating currents

The effects of trypsin on the gating current components, I_g(on) and I_g(off), associated with the opening and closing of channels are illustrated in Fig. 4. Trypsin considerably reduces the size of gating signals without producing appreciable distortions of their time-course (Figs. 4 a and b). In addition, the total charge associated with the 'on' traces, Q_{on}, decreases with a rate, ν_g, comparable

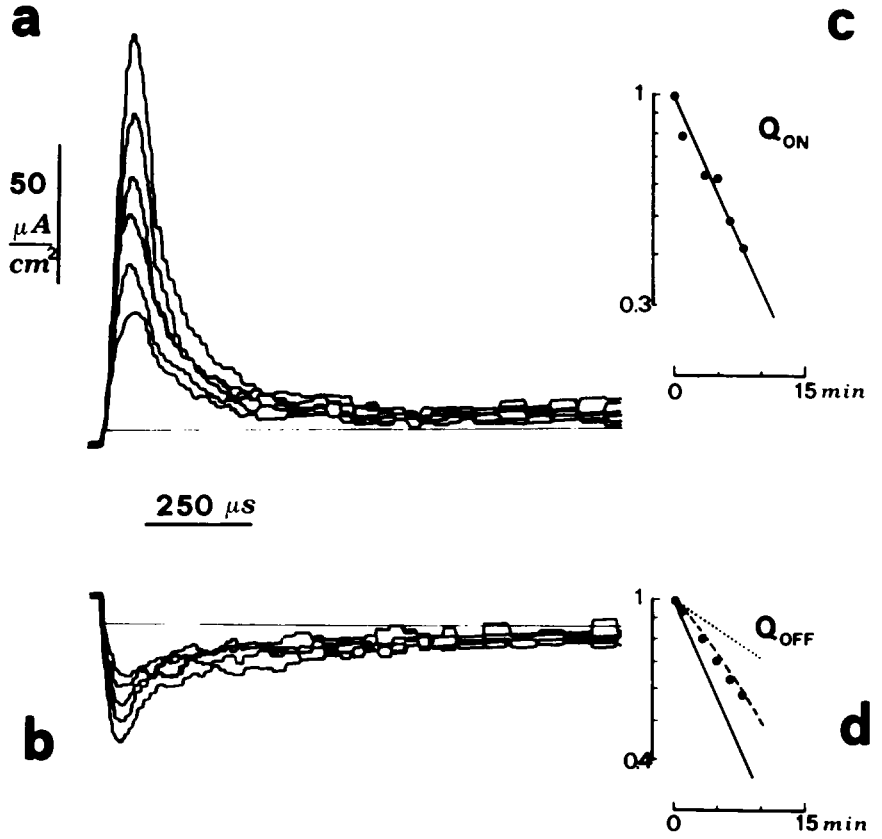

Fig. 4. Effects of trypsin on gating currents. (a) 'on' gating currents recorded by repeating four times a P/4 pulse procedure described in Methods. Test potential: +64 mV from −80 mV. Control pulses started from −128 mV. Traces were obtained at various time intervals during internal perfusion with 2 mg/ml trypsin Miles. (b) 'off' gating currents recorded on returning to −80 mV after 2 ms at +64 mV. (c) and (d) Time-course of gating charge movements associated with the 'on' and 'off' I_g components. Solid curves are straight lines with a slope of 1/9 min^{-1}. The dotted line has a slope of 1/31 min^{-1}. The dashed curve is derived by assuming that: (i) trypsin acts independently on the activation and inactivation process, (ii) the removal of sodium inactivation and the reduction of charge immobilization proceed at the same rate, and (iii) 2/3 of gating charges are immobilized. Under these conditions, $Q_{off}(t)$ can be expressed as follows:

$$Q_{off}(t)/Q_{off}(0) = \{2\cdot[1-\exp(-\nu_h t)]+1\}\exp(-\nu_g t) \quad (1)$$

where $Q_{off}(0)$ is the total charge displaced before the enzyme action; ν_h and ν_g are respectively the rate of removal of charge immobilization and gating charges. The dotted curve is obtained by substituting $\nu_h = 1/31$ min^{-1} and $\nu_g = 1/9$ min^{-1} into Eqn. 1. Q_{on} and Q_{off} values at time zero were 23.8 and 8.2 nC/cm^2, respectively. Axon 1-17JL0. In: 350 Cs. Out: Tris-SW + $3\cdot10^{-7}$ M tetrodotoxin. $T = 13°C$.

to that of g_l ($1/9$ min^{-1}), while Q_{off} falls with a rate of nearly $1/14$ min^{-1} (Figs. 4 c and d). On five axons ν_g averaged $1/9.6 \pm 1/0.7$ (mean ± S.E.), which is close to the average rate of removal for g_l derived from Fig. 5, $1/10.1 \pm 1/1.2$. Incidentally, because of the progressive increases of leakage levels (comparable to the amplitude of gating currents) experiments like that of Fig. 4 normally lasted 5 to 8 min. Consequently, the removal of gating changes and the decrease of peak g_{Na} can only be compared at the very beginning of tryptic action.

In all the experiments, Q_{off} was always found to decrease more slowly than Q_{on}. This may be due to the parallel action of trypsin on the activation and inactivation gates of Na^+ channels. Q_{off} is known to increase upon removal of steady-state Na^+ inactivation because of the reduced immobilization of gating charges [23]. Thus in the case of Fig. 4, if trypsin acted simultaneously on

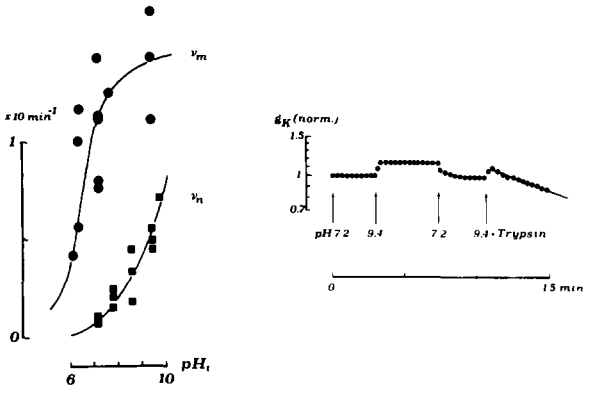

Fig. 5. (Right) Standard procedure for the determination of the rate of action of trypsin as a function of pH_i. Dots are normalized values of g_K as determined from the current tails measured at the end of a step depolarization to +100 mV lasting 8 ms. Repolarization to −70 mV. Arrows indicate the changes of internal solution. pH 7.2 refers to the standard internal perfusate: 400 K. pH 9.4 is 400 K with potassium phosphate substituted by potassium glutamate buffer. Trypsin at a concentration of 2 mg/ml was added immediately before test measurements. The straight line has a slope of approx. $1/18$ min^{-1}. Axon 2-JN260. Out: artificial sea water + $3 \cdot 10^{-7}$ M tetrodotoxin. $T = 13$°C. (Left) pH-dependence of the rate of decrease of peak g_{Na} (ν_m, circles) and g_K (ν_n, squares) during trypsin digestion. Each dot is obtained from a different axon. Lines through experimental points are drawn by eye. In: 400 K for ν_n and 50 Na for ν_m. Out: artificial sea water + $3 \cdot 10^{-7}$ M tetrodotoxin for ν_n and 1/4 Na-SW for ν_m. $T = 13$°C.

Na^+ activation and Na^+ inactivation, Q_{off} is expected to decrease with a time-course intermediate between the decay of Q_{on} ($1/9$ min^{-1}) and the 3-fold increase of charges produced by the removal of Na^+ inactivation ($1/31$ min^{-1}). An attempt to account for this phenomenon is given in the legend of Fig. 4.

Rate of action of trypsin on Na^+ and K^+ conductance as a function of pH

To gain an insight into the mechanism regulating the enzyme-substrate reaction, the effect of internal pH (pH_i) on the rate of disappearance of functioning channels (ν) was studied. Fig. 5 (right) illustrates the procedure adopted to determine ν for potassium conductance (ν_n) at various pH_i. Conductance measurements usually started with a test of the effects of pH_i (second arrow from the left). After a short recovery to normal conditions the enzyme was applied at test pH_i (fourth arrow) for approx. 5 min, and ν_n determined from the slope of the conductance decrement. An identical procedure was followed to evaluate ν for sodium (ν_m).

The results obtained from a total of 27 axons are presented in Fig. 5 (left). Although the ν_m data scatter more than the ν_n, they appear to conform to an S-shaped titration curve with a pK_a of about 6.7. In contrast, ν_n increases steeply at high pH_i without showing any tendency to reach a plateau level up to pH_i 9.7. Tests at pH_i higher than 9.7 and lower than 6.2 were hampered by a strong increase of leakage currents.

Discussion

The main finding of the present work is that intracellular application of trypsin to voltage-clamped nerve fibres furnishes a new way of separating sodium from potassium currents. This approach has the advantages of abolishing Na^+ channels and their gates at nearly the same rate without affecting the time course of the potassium currents. In this connection, also the enzyme-mixture pronase has been found to affect preferentially Na^+ currents when intracellularly applied to squid axons, with only minor effects on the potassium system [6].

Two conclusions can be drawn from the one-to-

one correlation existing between the decrease of g_{Na} and Q_{on}. First, in agreement with previous findings [2,3] the major fraction of fast gating currents is related to the activation gate of the sodium channel. However, the possibility that part of the measured gating currents is due to charge movements related to macromolecular structures not belonging to the sodium channel [4,25] can not be ruled out a priori. Trypsin might cleave sodium channels and other charged groups at a comparable rate. Second, in terms of a sequential model [23], trypsin very likely acts by modifying the resting closed state of the channel, thereby preventing the movement of gating charges and the permeation of ions through the pore. The alternative, that trypsin modifies intermediate channel-states leaving unaltered the preceding steps, would only produce a partial loss of 'on' gating charges as compared to the number of non-conducting channels. One would therefore expect ν_g to be smaller than ν_m, and not nearly the same, as observed in this work.

As suggested by the results of Fig. 5, the action of trypsin on membrane currents might reflect a different structural organization of Na^+ and K^+ channels. In particular, the close similarity between the pH-sensitivity of ν_m and the pH-dependence of the trypsin-catalyzed hydrolysis of readily accessible peptide bonds formed by lysine and arginine residues [18], would indicate the presence of positive charged groups at or near the inner side of the Na pore involved in the turning-on mechanism of the channel. Unfortunately, very little can be argued from the present data about the possible groups implicated in the activation machinery of the K^+ channel.

Acknowledgments

I am grateful to Drs. E. Wanke and P. Testa without whose help this project would have been impossible, to Drs. A. Maelicke and M. Lazdunski for encouraging suggestions and to Ms. O. Graffigna for secretarial assistance.

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