Morphology and Electrophysiological Properties of Squid Giant Axons Perfused Intracellularly with Protease Solution

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ABSTRACT   Squid giant axons were perfused intracellularly with solutions containing various kinds of proteases (1 mg/ml). Except for a 10 μ layer inside the axolemma the axoplasm was removed by a 5 min perfusion with *Bacillus protease*, strain N' (BPN'). The resting and action potentials were unchanged and the axon maintained its excitability for more than 4 hr on subsequent enzyme-free perfusion. After perfusion with protease solution for 30 min the axoplasm was almost completely removed. The excitability was maintained, but the action potential became prolonged and rapidly developed a plateau of several hundred milliseconds. The change was not reversible even when the enzyme was removed from the perfusing fluid. Two other enzymes, prozyme and bromelin, also removed the protoplasm without blocking conduction. Trypsin suppressed within 3 min the excitability of the axon. It is suggested that the proteases alter macromolecules in the excitable membrane and thus affect the shape of the action potential.

INTRODUCTION

If, as suggested, the membrane of the squid giant axon is composed of proteins as well as phospholipids (Tobias, 1955) and if proteins are involved in the excitable process, intracellular perfusion with proteases should affect the protein molecules of the membrane and thereby influence the excitability of the axon. In fact intracellular administration of various proteases has been shown to suppress irreversibly the excitability of the axon (Rojas and Luxoro, 1963; Tasaki and Takenaka, 1964 *a*; Tasaki, Singer, and Takenaka, 1965).

The present work describes the results obtained by intraaxonal perfusion with other proteases which remove almost all the axoplasm without causing loss of excitability. The duration of the action potential increased gradually when these protease solutions were introduced in the course of continuous perfusion. This change is regarded as an indication of an alteration of protein

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molecules in the membrane. A preliminary note has been published (Takenaka and Yamagishi, 1966).

MATERIAL AND METHODS

Live squid, *Doryteuthis bleekeri*, were obtained at the Marine Biological Station of Tokyo University, Misaki, Japan, between January and March. The giant axons, generally used without extensive cleaning, were 40–50 mm in length and 450–600 μ in diameter.

The experimental technique was basically similar to that of other investigators (Oikawa, Spyropoulos, Tasaki, and Teorell, 1961; Tasaki and Takenaka, 1964 *b*). The axon was mounted on a Lucite platform. A large outlet cannula, 280–350 μ in diameter and 35 mm in length, was first inserted longitudinally into the axon from a cut end until its tip passed the midpoint of the axon. An inlet cannula, 160 μ in diameter, was next pushed into the axon from the other cut end until its tip nearly reached the tip of the outlet cannula. The inside of the outlet cannula was then cleaned with a fine wire. When the bottle of perfusing fluid was raised, the pressure inside the inlet cannula increased and started the internal perfusion. The initial perfusing fluid was a 400 mM KF solution with an enzyme. The two cannulae were slowly retracted until their tips were 10–14 mm apart. After about 5 min from the onset of perfusion, the perfusing fluid was switched to an enzyme-free 400 mM KF solution. The bottle containing the perfusing fluid was about 40–60 cm above the level of the axon and the height was adjusted for a flow rate of 0.03 ml per 40 sec from the outlet cannula. The entire perfusion system was kept at 14–16°C.

Stimuli of 0.1 msec duration and twice threshold intensity were applied through an isolation unit to a pair of external platinum electrodes near the proximal end of the perfused zone. Propagated action potentials were recorded with a second pair of platinum electrodes near the distal end of the axon and displayed on one beam of a dual-beam oscilloscope. The intracellular recording electrode was a glass capillary of 60 μ outside diameter filled with 0.6 M KCl. Its impedance was reduced by a bright platinum wire placed inside the capillary. A large silver-silver chloride-KCl agar electrode was placed in the external medium to serve as a ground. The rate of rise of the spike was recorded by a differentiating circuit with a time constant of 25 μsec.

For measurement of the membrane resistance two silver wire electrodes were used as internal electrodes, one for recording and another for supplying current. The current electrode was made of 50 μ enamelled wire, with two bare portions of 5 mm near the tip. The recording electrode was twisted around the current electrode, and a region of 4 mm was bared midway between the two bare areas of the current electrode. Both electrodes were carefully coated electrolytically with silver chloride. As an external current electrode, an Ag-AgCl wire was dipped into the bathing fluid. The external potential electrode was a calomel electrode. Both internal electrodes were inserted through the outlet cannula into the perfusion zone. Inward current pulses delivered by the first wire were monitored by one oscilloscope beam. The potential variation produced by the current wire was simultaneously monitored by the other beam. Voltage-current curves were obtained by plotting the steady-state values for electrotonic potential against the intensities of applied current (duration of 50 msec).

The standard perfusing fluid contained 400 mM KF (400 KF) and was made by mixing two parts of 600 mM KF and one part of 12% glycerol solution (Table I). The pH of the solution was adjusted to 7.2 with Tris-HCl buffer. The external solution generally was natural seawater.

Four proteases were used in these experiments. (1) BPN' (*Bacillus protease*, strain N'), prepared from *B. subtilis* var. *biotecus*, A (Hagiwara, 1960), is commercially available as "Nagarse" (Nagase Co., Ltd., Tokyo, Japan). BPN' is a mixture of endopeptidases having a very wide specificity. BPN' also has amylase activity amounting to 2% of its protease activity. It attacks about one-third of the peptide bonds in casein and one-fourth of these in gelatin, whereas trypsin of the same proteolytic activity can hydrolyze only one-fifth and one-tenth, respectively. Many free amino acids are detected in the casein digestion mixture (Hagiwara, Nakai, Matsubara, Komaki, Yonetani, and Okunuki, 1958). The activity of the enzyme changes very little in the 6–10 pH

TABLE I
INTERNAL PERFUSING SOLUTION

	KF	Glycerol
	mM	mM
400 KF	400	500
100 KF	100	1250

range and is stable in the presence of certain salts, e.g., Na^+, Ca^{++}, and NH_4^+ (Hagiwara, 1960). (2) Prozyme prepared commercially from *Streptomyces* No. 1033 (Kyowa-Hatsuko Co., Tokyo, Japan). Prozyme is a mixture of proteases and has an exceptionally wide range of side-chain specificity. The activity of this enzyme is stable in the 7.0–8.0 pH range. (3) Bromelin prepared commercially (Hawaiian Pineapple Co., Hawaii, U. S. A., Lot 184). The enzyme is a mixture of proteolytic enzymes obtained from the pineapple plant (Smith and Kimmel, 1960). (4) Trypsin prepared commercially (Mochida Pharmacological Co., Tokyo, Japan). Each enzyme was mixed with the perfusing fluid (400 KF) in a concentration of 1–0.1 mg per 1 ml solution. These mixtures will be called 400 KF-enzyme.

In order to investigate to what extent the axoplasm was removed by the perfusion of proteolytic enzymes, both light and electron microscope studies were carried out. Axons were fixed with glutaraldehyde containing phosphate buffer (Sabatini, Bensch, and Barrnett, 1963), kept in a refrigerator (0–2°C) for about 1 hr, and then washed and stored in phosphate buffer solution until fixation with 1–2% OsO_4. After dehydration through an ethanol series the specimens were embedded in epoxy resin (Luft, 1961). For light microscopy, a large part of each block was trimmed and thick sections were cut with a glass knife on a Porter-Blum microtome and stained with toluidine blue. For

electron microscopy, thin sections of a smaller area in the same block were cut and stained doubly with uranyl and lead. Observations were made with a Hitachi HS 7 electron microscope (Hitachi Co., Tokyo, Japan).

RESULTS

1. *Removal of Axoplasm with BPN'*

The upper part of Fig. 1 represents an experiment in which the axon was perfused first with 400 KF-BPN' for 5 min and subsequently with 400 KF for
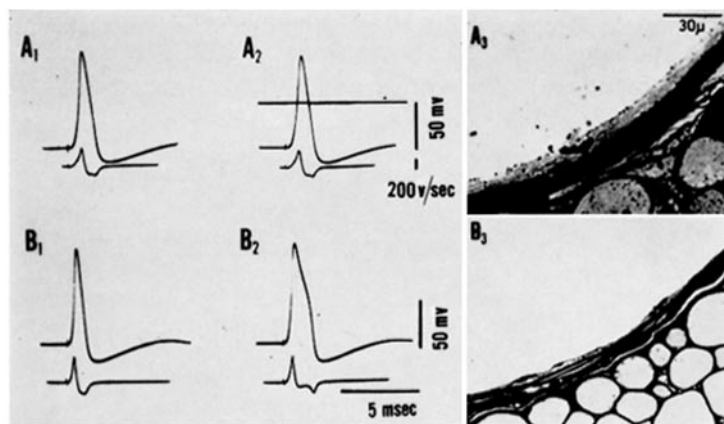

FIGURE 1.   Action potential and photomicrograph of the squid giant axon under intracellular perfusion with BPN'. The series A_{1-3} was obtained from the same axon which was perfused with BPN' for 5 min and afterwards with 400 mM KF solution for 25 min. A_1, at the beginning of perfusion as a control. A_2, after 30 min perfusion. Horizontal bar shows zero potential. A_3, preparation fixed at 30 min, just after recording A_2. The series B_{1-3} was obtained from another axon which was continuously perfused with BPN' for 30 min. B_1, at the beginning of perfusion as a control. B_2, after 30 min perfusion. B_3, fixed at 30 min, just after recording B_2. In the photomicrographs the axons were fixed with 5% glutaraldehyde, embedded in Epon, and after sectioning stained with toluidine blue.

25 min. A_1 shows the action potential immediately after starting the perfusion with the enzyme solution and A_2 shows the action potential 30 min later. The perfusing fluid flowed freely throughout the experiment. Immediately after recording the action potential in A_2, the axon was fixed with 5% glutaraldehyde. A light micrograph of this axon is shown in A_3. It can be seen that with the above procedure the shape and magnitude of the action potential did not change appreciably. The rate of change of potential, shown in the lower traces, did not change either. The resting potential remained constant at about 50 mv. The light micrograph in Fig. 1 A_3 shows that all the axoplasm was removed, except for a layer of about 10 μ next to the membrane.

The effect of a longer exposure to the enzyme solution is shown in the lower

part of Fig. 1. The axon was continuously perfused with 400 KF-BPN' for 30 min. The action potential in B_1 was obtained at the beginning of perfusion. The action potential in B_2 was obtained 30 min later. B_3 is a light micrograph of this axon, fixed immediately after recording the action potential in B_2. During the experiment the resting potential and the action potential amplitude were constant (55 mv and 110 mv, respectively). The duration of the action potential increased from 1 to 2 msec. The lower trace shows the rate of potential change and the maximum rate of rise changed little. The light micrograph in B_3 shows that little axoplasm remained after the perfusion. The electron micrograph in Fig. 2 (lower part) was obtained from the same preparation as in Fig. 1 B_3. It shows that the axolemma still remained and was covered internally only by a small trace of electron-absorbing substance. The Schwann cell layer showed marked vacuolization. These results indicate that action potentials can be obtained from an axon after practically complete removal of the axoplasm.

2. Configuration Change in the Action Potential Caused by Continuous Perfusion with BPN'

The records in Fig. 3 were obtained from an axon continuously perfused with 400 KF-BPN'. Figs. 3 A and 3 B show the action potential recorded immediately and 30 min after the onset of perfusion, respectively. C and D show the action potentials recorded with two different sweep speeds 35 min after the onset of perfusion. As perfusion continues, the duration of the action potential increases, while its amplitude and the resting potential remain almost constant. The prolonged action potential had an initial peak followed by a long pleateau. This configuration, illustrated completely by Fig. 3 D, is similar to that of the cardiac action potential (Draper and Weidmann, 1951). The maximum rate of potential change (see lower trace) was almost constant (A and B), but decreased gradually later (C and D).

The action potential duration is plotted against the time of perfusion in Fig. 4. The duration was measured at a level that was less negative than the resting level by 10% of the spike height. In the experiment of the upper graph the axon was perfused continuously with 400 KF-BPN'. The process showed three stages. During the first stage (stage A) the duration of the action potential increased gradually until it reached about 5 msec. At the end of this stage the duration increased very rapidly and attained a peak of about 400 msec (stage B). That was maintained for about 20 min. At the beginning of the third stage (stage C) the duration rapidly became smaller. In the second part of stage C it still decreased, but more gradually. In stages A and B the amplitude of the action potential was almost constant; in stage C it decreased gradually. The rate of rise of the potential was about the same as that of the control in stage A, decreased to about two-thirds the control value in stage
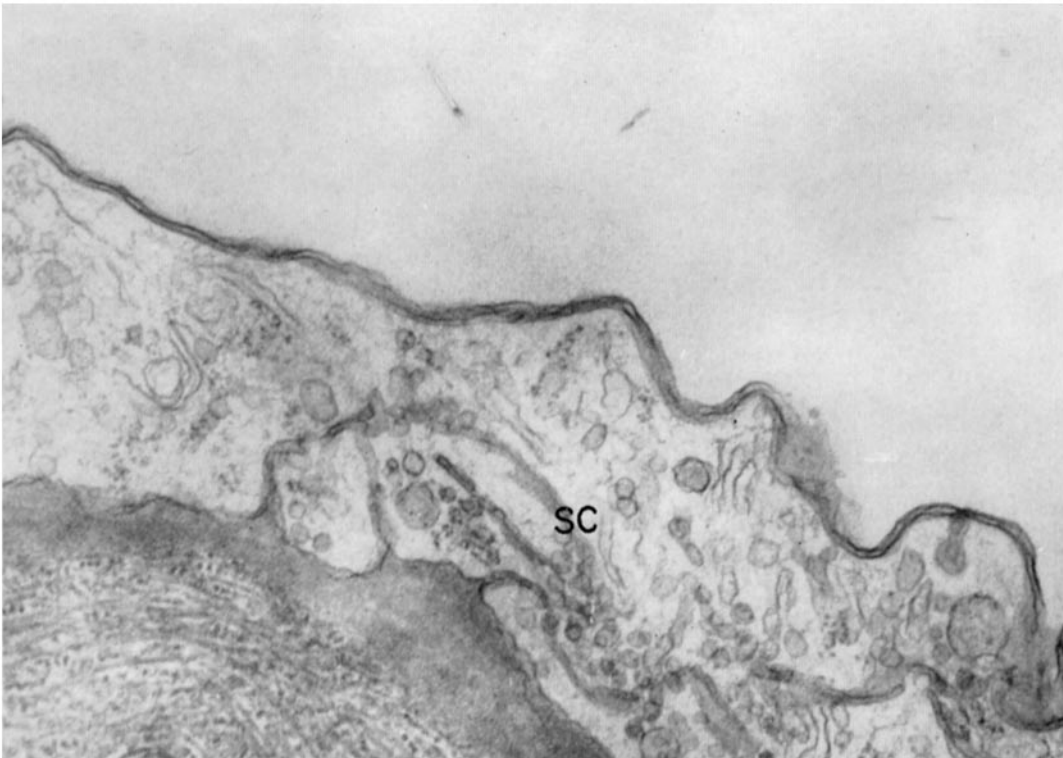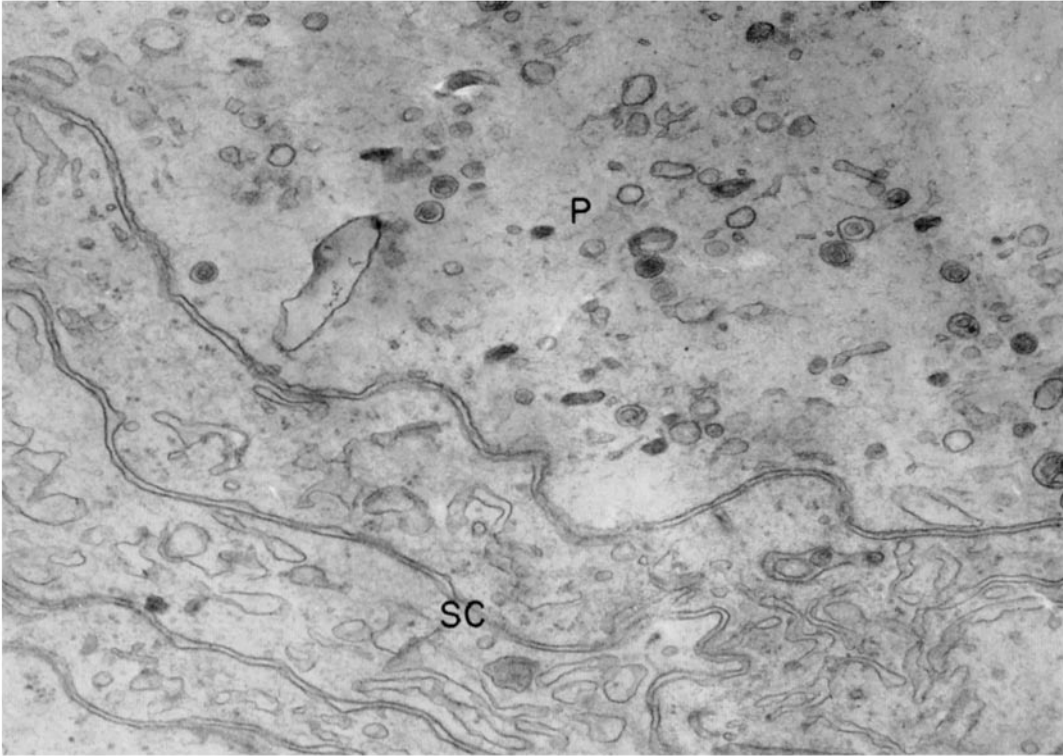
B, and decreased even further in stage C. In stages A and B the resting potential was the same as that of the control; in stage C, it decreased gradually. Stage C could be considered as a deteriorated stage. This axon maintained excitability for 95 min. The lower graph of Fig. 4 represents data on an axon perfused first with 400 KF-BPN' for 5 min and subsequently with 400 KF continuously. The duration of the action potential changed very little. The action potential amplitude and the resting potential did not change at all. This axon maintained its excitability for more than 4 hr.

3. Potential Change by Continuous Perfusion with Other Enzymes

The effects of the proteases, prozyme and bromelin, on the axon were also examined. Fig. 5 shows a series of action potentials which were obtained by continuous perfusion with prozyme, immediately after starting the perfusion (A) and 10 min later (B). C shows the initial phase of the prolonged action potential observed after 15 min of perfusion and D shows the entire prolonged action potential observed after 20 min. The duration of the action potential which increased with the duration of perfusion, reached about 1 sec after 15 min perfusion. This prolonged action potential persisted for about 40 min. Thereafter the axon gradually deteriorated. The amplitude of the action potential decreased by about 5 mv from A to B, remained at about the same size through C and D. The resting potential was also almost constant and close to 50 mv. Perfusion with prozyme produces a prolonged action potential earlier than does perfusion with BPN' (e.g 15 min vs. 30 min), and the stage of the prolonged action potential is more durable with prozyme than with BPN'. During the prozyme perfusion we often observed an oscillation of the spike on the plateau phase of the prolonged action potential (Fig. 5 C).

Bromelin also causes prolonged action potentials, but the survival time of the axon is usually shorter than with BPN' or prozyme. When perfused with trypsin the survival time of the axon was only 2 or 3 min. No prolonged action potentials were produced. Decreasing the trypsin concentration to as low as 0.01 mg per 1 ml solution did not increase the survival time.

4. Effects of Proteases Acting from the External Medium

BPN', prozyme, or trypsin was added separately to the external fluid at a concentration of 1 mg/ml. The resting potential, the excitability, and the

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FIGURE 2. Electron micrographs of perfused squid axons which were fixed with 5% glutaraldehyde followed by osmium fixation and embedding in epoxy resin. Above, intact axon including protoplasm (P). The Schwann cell (SC) is closely adjacent to the axon. The plasma membrane is closely invested by the membrane of a Schwann cell. Below, axon perfused with BPN' for 30 min. The axoplasm is mostly removed except for a trace of axoplasm tightly bound to the membrane. The axon membrane is intact and paralleled by a Schwann cell membrane. × 40,000.
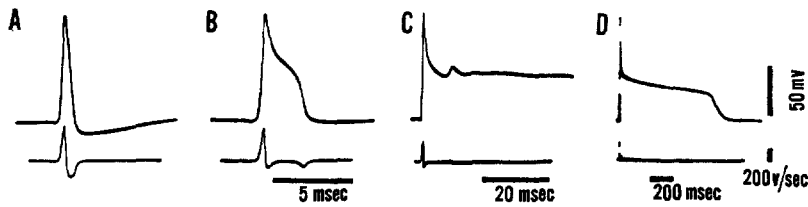
FIGURE 3.   Changes in the action potential due to the continuous perfusion of BPN'. A, action potential recorded at the beginning of perfusion. B, 30 min after starting perfusion. C and D, 35 min from the onset of perfusion taken with different sweep speeds. After the action potential duration lengthened up to 5 msec, the plateau phase suddenly occurred. The level of the plateau is approximately 0 mv in most cases.

action potential configuration of the axon remained unchanged, even after 10 hr.

5. *Voltage-Current Curves of Perfused Membrane in the Resting State*

The voltage-current relations of the axon membrane during the perfusion of protease and varied potassium concentrations were studied. Fig. 6 shows an example of voltage-current relations on perfusion with 400 KF and with
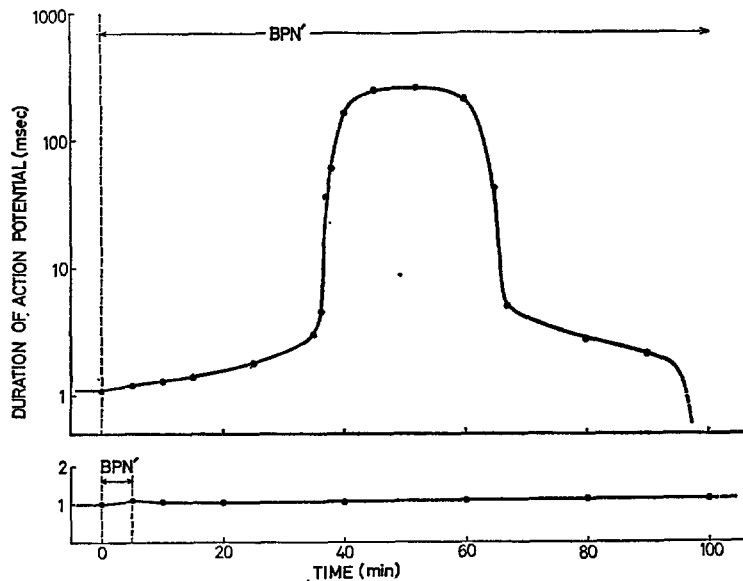

FIGURE 4.   Relation between the duration of the action potential and the time course of intracellular perfusion. Upper trace, an axon continuously perfused with 400 KF-BPN'. The prolonged action potential suddenly occurred and was observed for 30 min. Lower trace, an axon first perfused with 400 KF-BPN' for 5 min and after that with 400 KF. No change in the shape of the action potential was observed and the excitability was maintained for more than 3 hr.
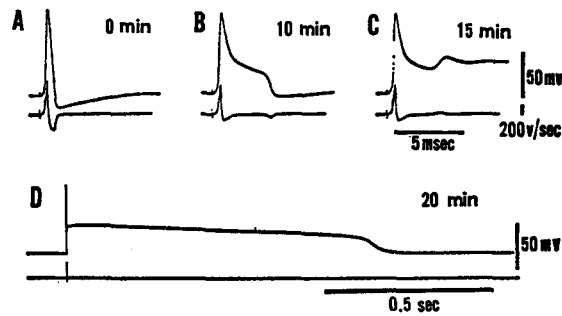
FIGURE 5. Changes in action potential due to continuous perfusion with prozyme. A, action potential recorded at the beginning of perfusion. B, 10 min later. C, after 15 min perfusion. A prolonged action potential occurred. D, the typical prolonged action potential observed after 20 min prozyme perfusion. The lower traces show the rate of rise in the action potential.

100 KF. In this case the sodium chloride in the external medium was replaced by choline chloride to avoid the discharge of impulses upon depolarization. The slope resistance increased with hyperpolarization and decreased with depolarization with weak intensity of polarizing current when 400 KF was inside the axon. With the internal potassium concentration lowered to 100 mM, the voltage-current relation became less steep. Delayed rectification developed at much greater depolarizations with 100 KF inside than with 400 KF. At large hyperpolarizations there was almost no difference between the
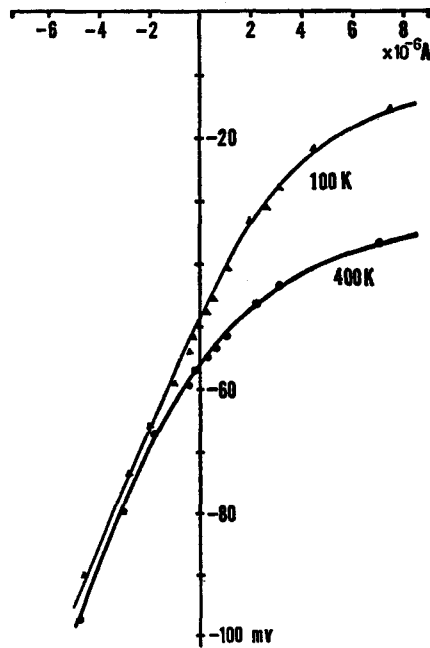

FIGURE 6. Voltage-current relation in an axon perfused internally with 400 mM KF and with 100 mM KF in Na-free choline seawater.

slope resistances when perfusing with these two internal solutions. The above results are similar to the voltage-current relation obtained by using potassium sulfate or chloride solutions (Narahashi, 1963).

In Fig. 7 A voltage-current relations in 400 KF with and without BPN' are illustrated. In this experiment the axons were initially perfused with 400 KF and then with 400 KF containing BPN'. In the latter case measurements were done between 30 and 60 min from the onset of enzyme perfusion and after producing the prolonged action potentials. The membrane resistance at the resting potential was almost the same in both cases. In Fig. 7 B, voltage-
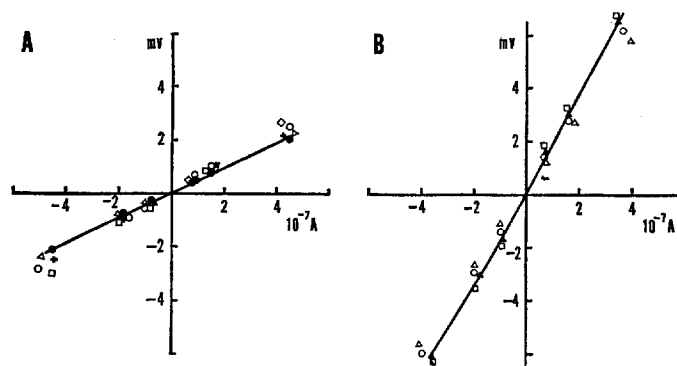

FIGURE 7. Voltage-current relation in the axons perfused internally with potassium solutions and BPN'. A, 400 KF (●) and 400 KF-BPN' (○, △, □, ×). B, 100 KF (▲) and 100 KF-BPN' (○, △, □). No changes in membrane resistances were observed after the perfusion with BPN'.

current relations in 100 KF with and without BPN' are illustrated. We applied a weak current to the membrane during the stage of prolonged action potentials. The membrane resistance was little changed in three axons examined when the enzyme was added. These results show that the membrane resistance at the resting potential is not changed markedly by the enzyme.

6. *Effects of Ca++ and Mn++ from External Medium on Prolonged Action Potential*

The duration of the action potential is strongly affected by the external calcium concentration. The records of Fig. 8 show one example, in which the concentration of sodium ions in the external medium was kept constant at 300 mM and the concentration of calcium ions was changed. This Ca saline was obtained by replacing the glycerol portion of Na glycerol solution with an osmotically equivalent amount of $CaCl_2$. The axon was initially perfused with 400 KF-prozyme for 10 min and later continuously perfused with 400 KF. In Fig. 8 A the external medium contained 33.3 mM $CaCl_2$ in artificial seawater. The duration of the spike was 365 msec. In the presence of 66.6 mM

$CaCl_2$ (B), the duration of the spike decreased from 365 to 120 msec. On reducing the $CaCl_2$ to 33.3 mM (C), the duration of the action potential increased again to 275 msec. When a fluid containing 200 mM $CaCl_2$ was applied externally (D), the duration of the spike became only 35 msec. In E the external fluid was again changed to the artificial seawater containing 33.3 mM $CaCl_2$ and the duration again increased. Thus, the duration of the prolonged action potential is reversibly decreased with an increase of Ca^{++} concentration.
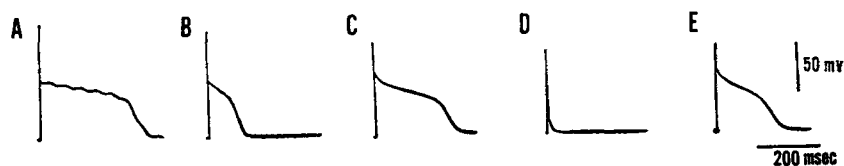

FIGURE 8. Effect of Ca^{++} on the duration of the prolonged action potential. A, 33.3 mM $CaCl_2$. B, 66.6 mM $CaCl_2$. C, 33.3 mM $CaCl_2$. D, 200 mM $CaCl_2$. E, 33.3 mM $CaCl_2$. Ca^{++} concentration in the outer solution affected the plateau phase. 200 mM $CaCl_2$ abolished the plateau phase reversibly.
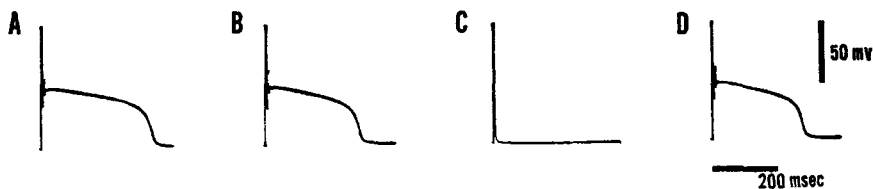

FIGURE 9. Effect of Mn^{++} on the duration of the prolonged action potential. A, 0 mM $MnCl_2$. B, 16 mM $MnCl_2$. C, 32 mM $MnCl_2$. D, 0 mM $MnCl_2$. 32 mM $MnCl_2$ abolished the plateau phase reversibly.

The effects of Mn^{++} in the external fluid on the duration of the prolonged action potential were also studied. In Fig. 9 the concentrations of Mn^{++} were 0, 16, 32, and 0 mM in records A, B, C, and D, respectively. The duration of the action potential measured at the resting level decreased from 380 to 310 and 15 msec as the Mn^{++} concentration was increased. In D, when Mn^{++} was removed from the external solution, the prolonged plateau of the action potential reappeared. The effect of Mn^{++} in shortening the duration of the prolonged action potential was about six times stronger than the effect of calcium.

7. Combined Effects of K^+ Concentration and Enzyme on Prolonged Action Potential

As is well-known, internal perfusion with low potassium solutions reduces or eliminates the after-hyperpolarization and slows the rate of fall of the spike

(Narahashi, 1963; Baker, Hodgkin, and Meves, 1964; Moore, Narahashi, and Ulbricht, 1964; Adelman, Dyro, and Senft, 1965 *a*). This tendency was enhanced enormously after treating the axon with an enzyme solution. The axon in Fig. 10 was initially perfused with 400 KF and exhibited the action potential shown in A. After subsequent perfusion with 100 KF for about 10 min it exhibited the action potential shown in B. The falling phase of the action potential was slowed by lowering the internal potassium concentration and the undershoot disappeared. The internal perfusing solution was then switched
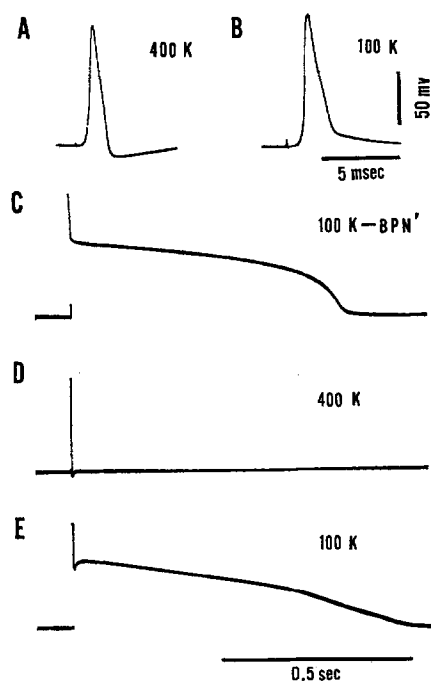

FIGURE 10. Combined effects of BPN' and potassium concentration of the action potential duration. A, recorded at the beginning of perfusion with 400 KF. B, perfused with 100 KF. C, perfused with 100 KF-BPN'. D, perfused with 100 KF. Description in text.

to 100 KF-BPN', and the action potential duration increased gradually. After about 10 min the duration was 0.7 sec (C) and stayed constant. Next, we changed the perfusing solution to 100 KF: the shape of the action potential remained as in C for about 1 hr. When the perfusing solution was changed back to 400 KF, the duration of the action potential suddenly became shorter (5 msec) and is shown in D. When the perfusing solution was again changed to 100 KF, the duration of the action potential became longer suddenly and is shown in E. Table II shows the relation between potassium concentration and the time at which the action potential is prolonged by perfusing with BPN'. The tendency to produce a prolonged action potential is enhanced when a low potassium internal solution is used as enzyme solvent. With 400 KF-BPN' internal perfusion, about 30 min are required to produce a prolonged action potential; with 100 KF-BPN', 10 min are enough. These results

shown in Fig. 10 and Table II suggest that the production of a prolonged action potential depends on the combined effect of the internal potassium concentration and the enzyme action upon the membrane structure.

DISCUSSION

Protease Perfusion Several workers have perfused squid giant axon with proteases (Tasaki and Takenaka, 1964; Tasaki et al., 1965). Trypsin and chymotrypsin blocked the excitability of the axon within 2.5–6 min at concentrations of 1 mg/ml, or less. With papain and ficin excitability persisted for up to 20 min, but not more. The BPN' and prozyme used in these experiments did not abolish the excitability for periods of up to 4 hr.

TABLE II

Axon No.	Enzyme solvent	Action potential	Resting potential	Maximum spike duration	Time at which the action potential is prolonged by perfusing enzyme
		mv	*mv*	*msec*	*min*
4	400 KF	105	80	850	35
13	400 KF	100	83	350	25
14	100 KF	110	80	500	15
16	100 KF	125	75	1500	10
28	400 KF	140	66	500	30
46	400 KF	115	83	160	20
50	400 KF	115	75	160	30
51	400 KF	116	80	1200	40
58	100 KF	110	70	360	15
59	400 KF	125	60	350	30

The first purpose of the perfusion of the giant axon with an enzyme solution was to eliminate the axoplasm so as to maintain a good flow of perfusion fluid. BPN' or prozyme proved to be the most suitable in this respect. The second purpose of the intraaxonal perfusion with enzyme solutions is to determine whether the action potential is still produced in an axon with almost no axoplasm. We have found that action potentials can indeed be produced from such axons, although their duration is prolonged. A third purpose of perfusing with proteases is to observe what changes occur in the excitable membrane. When the axon was perfused with enzyme after removal of the axoplasm, the duration of the action potential was gradually increased and reached several hundred milliseconds or even a few seconds. This suggests that protein molecules in the membrane play an important role in the excitability and are gradually changed by the protease.

It is very likely that the proteolytic enzymes and not others produced the effects reported here. Indeed, even though BPN' contains some amylase, the latter contributes only 2% of the total enzyme activity. Moreover, prozyme

and papain contain only proteolytic enzymes, and produce the same kind of prolonged action potential (Tasaki and Takenaka, 1964 a; Tasaki, 1968).

In general the proteolytic enzymes which we used can be divided into three groups, based on the maintenance of excitability in the perfused axon membranes. Type 1 is the trypsin-chymotrypsin group, type 2 is the papain-ficin-bromelin group, and type 3 is the BPN'-prozyme group. With type 1 the axon survives only a few minutes of perfusion. With type 2 the prolonged action potential is observed and its excitability continues for 10–30 min. With type 3, if the axon is perfused, the excitability remains unchanged for more than 1 hr although the configuration of the action potential changes to a prolonged spike in the later stage.

The question arises as to why the excitability of the axon disappears with proteases like trypsin or chymotrypsin, while it remains for 2 or 3 hr with BPN' or prozyme. Trypsin is an endopeptidase with a preferential specificity towards the peptide bond involving the carboxyl groups of the arginine and lysine (Desnuelle, 1960 a). Chymotrypsin also has a preferential specificity towards the peptide linkage involving the carboxyl groups of aromatic amino acids (Desnuelle, 1960 b). BPN' and prozyme, on the other hand, are endopeptidases of bacterial origin which have a wide specificity and whose proteolytic activity is usually higher than that of trypsin or chymotrypsin. It is interesting to note that the excitability of the axon was maintained during perfusion with BPN', which has a wide specificity, but not with trypsin which has a narrow specificity for protein molecules. It hardly seems justifiable, however, to speculate on these matters at present, since we have no information about the protein molecules in the membrane.

Prolonged Action Potential   It is possible to change the normal action potential of a squid giant axon into a prolonged action potential by several experimental procedures. Intracellular injection or perfusion of tetraethylammonium (TEA) ions and internal perfusion with dilute solutions of various alkali metal ions prolong the action potential in the squid giant axon (Tasaki and Hagiwara, 1959; Tasaki et al., 1962; Adelman et al., 1965 a; Baker et al., 1964; Armstrong and Binstock, 1965; Freeman et al., 1966). In our work prolonged action potentials were produced by internal perfusion with proteolytic enzymes. A distinct difference between the cases of TEA and enzyme is that the prolonged action potential produced by TEA returns to the normal short duration when TEA is washed out, while the prolonged action potential produced by enzymes remains prolonged after the enzymes are removed. Also, the prolonged action potential is not evoked until the axoplasm has been removed almost completely by the perfusion of enzymes. This suggests that some chemical linkages of the protein molecules in the membrane were altered by the enzyme. It is notable that even when some change in the structure of the membrane was presumably going on, no change in the mem-

brane resistance was observed. On the other hand, the reversible action of TEA suggests that there was no conformational change in the axon membrane.

The data of Fig. 10 indicate that a cooperative action exists between the action of the enzyme and the low internal potassium concentration for producing the prolonged action potential. The perfused enzyme appears to change the structure of the axon membrane slowly and irreversibly in a direction to induce the prolonged action potential. The reduced internal potassium (100-K) accelerates the initiation of the prolonged action potential. However, its effect is temporary and reversible, and is probably caused by the fact that the driving force for outward current is smaller with the low potassium perfusion when potassium activation occurs. When the internal potassium concentration is increased by perfusing with 400 mM KF, the duration of the spikes is shortened, but eventually the enzyme action also overcomes this driving force.

The prolonged action potential can also be explained by postulating changes in the mechanism controlling the ion permeability (Baker et al., 1964; and Adelman, Dyro, and Senft, 1965 *b*; Hodgkin and Katz, 1949), but the reason for the hypothetical ionic permeability change induced by the enzyme perfusion is still unknown.

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