*MEMBRANE POTENTIALS, RESISTANCE, AND ION PERMEABILITY IN SQUID GIANT AXONS INJECTED OR PERFUSED WITH PROTEASES**

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Several attempts have been made to produce known structural changes in cells by the use of enzymes and other agents as chemical dissectors and to determine the effects on functional activity.[1–7] Thus it has been found that external application of proteases seems not to interfere with normal permeability of red cells,[1] and with electrical function of squid, lobster, or frog axons.[2–6] It should be recalled, however, that externally applied protease does produce injury potentials in muscle.[5] External application of phospholipases, on the other hand, has shown that cell surface phospholipid integrity is essential for maintenance of normal resistance, capacity, and potentials in axons.[2–5]

Intracellular microinjection of protease destroys cell integrity of amoeba[7] and blocks excitability of the squid giant axon without apparent interference with resting potential.[8] This last result indicates that normal sodium and potassium permeability and membrane resistance are maintained. Since phospholipids in the membrane appear to be mandatory for normal permeability, resting potential, and resistance, protein seems to be somehow involved in modifying these phospholipids to permit the shift in the axon from the resting to the excited state.[9]

The present experiments were undertaken to determine the effect of internally applied proteases upon the axon membrane potentials and resistance, and sodium and potassium permeability.

Materials and Methods.—Experiments were done with the giant axon of the squid *Loligo pealii* at the Marine Biological Laboratory, Woods Hole, Massachusetts. Most experiments were performed with axons of about 350 μ in diameter.

Microinjection and perfusion procedures: Micropipettes (50–100 μ external diameter) were introduced longitudinally into the axon from one cannulated end following the procedure described by Oikawa *et al.*[10] Test solutions were injected or perfused along the axon as the pipette was slowly withdrawn. Resting and action potentials, as well as current-voltage relationships, were determined at different points of the axon by the use of two microelectrodes (3 *M* KCl filled glass microelectrodes, 0.5–5.0 *M* Ohm resistance).

Current-voltage curves: By plotting steady values for electrotonic potentials against intensities of the applied square pulse of current, these curves were obtained. Since the current spreads from the tip of the current electrode, membrane resistance per square centimeter of cell surface could not be measured. The change in resistance was estimated computing ratios of the slopes of current-voltage relationships at a given voltage, for the same axon and for roughly the same distance between the microelectrodes (range from 100 to 300 μ).

Na^{24} and K^{42} effluxes: Labeled sodium or potassium was injected into the axon

together with the test solution. The giant fiber with both ends tied was mounted in a glass holder and the amount of radioactivity accumulated in the external sea water was collected every 10 min.

Enzymes: Trypsin, chymotrypsin, and papain were several times crystallized and salt-free (Sigma Chemical Co.). They were dissolved in 0.5 *M* KCl, PO_4^{3-} 0.03 *M*, pH 7.4, triple-glass distilled water being used as solvent. They were kept at 0°C, and solutions were not stored for more than 3 days.

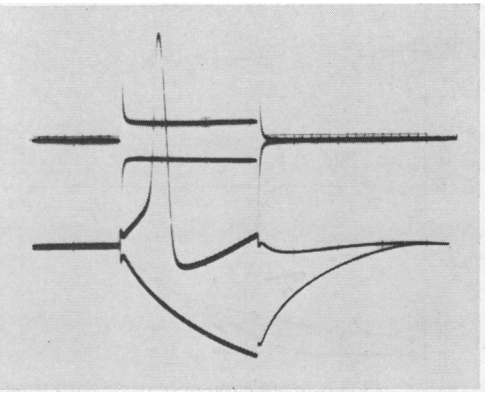

FIG. 1.—Membrane response recorded after microinjection. Axon 20, length injected with trypsin 23 mm, diameter 380 μ, amount injected $0.35 \cdot 10^{-3}$ ml. Depolarizing current 4μA. Hyperpolarizing current 5 μA.

Results.—All the experiments reported in this communication were performed using trypsin. A few experiments were done with α, β, and γ chymotrypsin and papain. The results obtained with this second group of enzymes were essentially similar to those observed in the case of trypsin.

Membrane potentials: The amount of protease injected was computed measuring the displacement of an air bubble in the pipette as this was slowly withdrawn from the interior of the axon. Figure 1 shows a membrane response recorded 5 min after the microinjection (axon 20, resting potential 52 mV, spike height 103 mV, undershoot 8 mV, threshold 2 μA). One sees that the membrane seemed not to be injured during the microinjection (50 experiments). Fibers with resting potential smaller than 50 mV and action potential smaller than 85 mV after the injection were discarded. A few exceptions were made in the case of low temperature experiments in which the range was 48–54 mV for the resting potential.

The effect of trypsin on membrane potentials at different times after injection is shown in Figure 2. It was found that after the action potential was almost abolished (25% of its original value) and the resting potential still 70 per cent of its original value, the axoplasm was almost liquefied. It could be easily removed from the interior and replaced with artificial solution (3 experiments). Removal of the axoplasm containing trypsin did not affect the last stage of the membrane potentials. That is to say, the axon was unexcitable, and the resting potential persists for longer times (around 2 hr) and in one case anodal break excitation was observed. Figure 3 shows the action potential at the end of the hyperpolarizing current (axon 27, resting potential 43 mV).

It should be pointed out that the temporal sequence of events after trypsin microinjection changes considerably from one axon to another. The volume injected is a fraction of the total intracellular space. Therefore, there is a dilution factor represented by

$$\frac{r_p^2 \, l_p}{r_a^2 \, l_a}$$

in which r_a and l_a are radius and length of the injected axon, r_p is the inner radius of

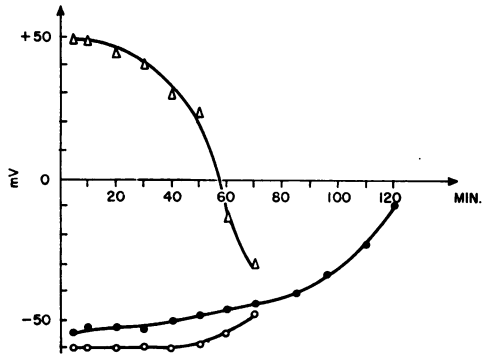

FIG. 2.—Trypsin effect on potentials with time. Axon 38, length injected with trypsin 25 mm, diameter 405 μ, amount injected $0.3 \cdot 10^{-3}$ ml. Triangles represent height of the action potential, filled circles represent resting potential, and open circles represent undershoot. After 60 min, several impalements were done at different points of the axon. The average resting potential was 41 mV (5 impalements). Temperature 20°C.

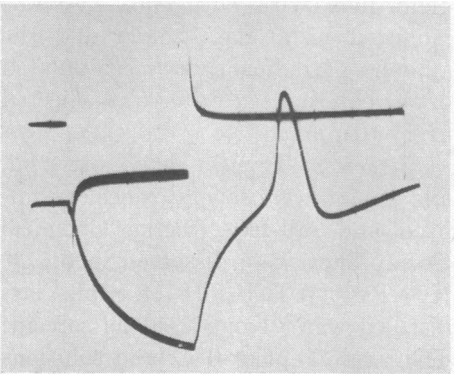

FIG. 3.—Anodal break excitation after removal of proteases. Axon 27, after 65 min the fiber was unexcitable. The axoplasm containing trypsin was removed and anodal break excitation observed at the end of a 38-μA hyperpolarizing pulse of current. See text.

the pipette, and 1_p the length of the solution column injected. For example, for axon 5, $r_a = 200\ \mu$, $1_a = 23$ mm, $r_p = 40\ \mu$, and $1_p = 28$ mm, the dilution factor was 0.05. In this case the final concentration of protease in the interior of the fiber was 0.05 mg/ml. It was found that the time required to reduce action potential to 25 per cent of its original value was correlated with the dilution factor, the time required being smaller, the greater the dilution factor was (for 8 experiments the range of variation was 10–80 min).

Membrane resistance: Current-voltage curves determined after trypsin microinjection are shown in Figure 4. The vertical axis represents the applied current, and the horizontal axis the steady change in membrane potential (axon 21, resting potential 55 mV, spike height 101 mV, undershoot 6 mV, distance between the

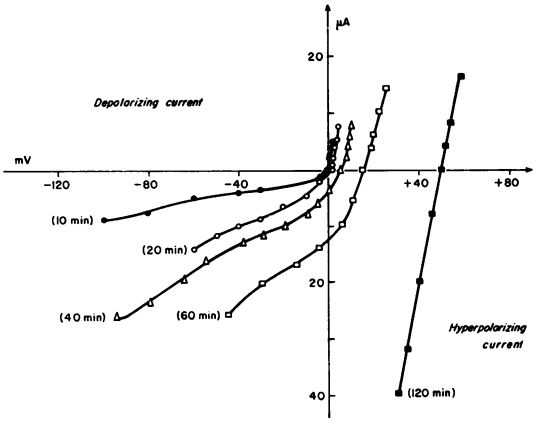

FIG. 4.—Current-voltage curves after trypsin microinjection. Axon 21, length injected with trypsin 22 mm, diameter 395 μ, amount injected $0.28 \cdot 10^{-3}$ ml. Horizontal axis represents displacement of the resting potential by either depolarizing or hyperpolarizing current.

microelectrodes 100–250 μ, several impalements). In this axon no action potential could be recorded after 50 min. At this time the resting potential was 43 mV.

In evaluating this result one should consider the fact that the membrane changes its dynamic resistance in time. The ratio of the slope of the current-voltage curve at −60 mV, 10 min after the microinjection, to the slope after 60 min is 0.8 when the test solution is the solvent and 0.3 when the test solution contained trypsin. This last figure represents the average of 4 experiments in which the dilution factor was of the same order of magnitude (around 0.1). It can be concluded, therefore, that when the resting potential is reduced to 70 per cent of its original value, there is also a decrease in membrane resistance.

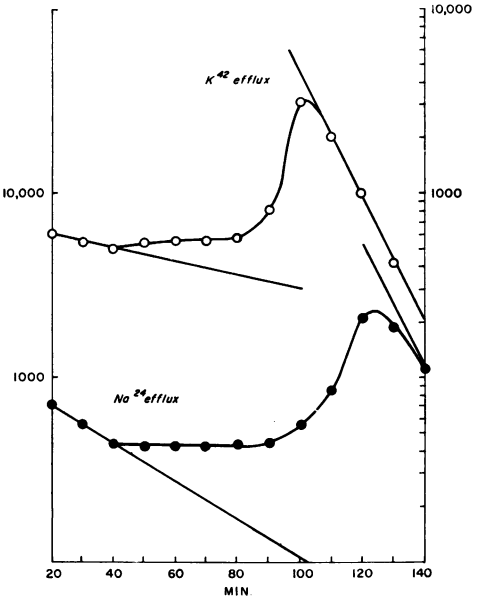

FIG. 5.—Na^{24} and K^{42} effluxes. Axon 41, length injected with trypsin 26 mm. Amount injected $0.4 \cdot 10^{-3}$ ml. Na^{24} outflux measured in cpm over background (left vertical axis). Axon 48, length injected with trypsin 24 mm. Amount injected $0.48 \cdot 10^{-3}$ ml. K^{42} outflux measured in cpm over background. In both cases horizontal axis represents time in minutes after trypsin microinjection. Temperature in both cases around 16°C.

Na^{24} and K^{42} effluxes: After knowing Na^{24} and K^{42} effluxes in fibers injected without protease, the data shown in Figure 5 were obtained (4 experiments). The first line was drawn with the slope found in the control experiments to fit the experimental points. After 40 min there is an increase in both sodium and potassium outfluxes.

There is an excellent agreement and consistency between the temporal sequences of trypsin-induced changes determined by measuring resting and action potential, membrane resistance, and membrane permeability to sodium and potassium. However, Na^{24} and K^{42} outfluxes were measured in different axons and only external stimulation and recording was possible. No attempts have been made to record simultaneously potentials and ion fluxes. Therefore it is not possible to conclude which ion is affected first.

Controls: Two types of experiments show that protease effect in membrane potentials, resistance, and permeability is due to enzymatic effect upon protein:

(1) Eight microinjections of solute alone did not show the described effect. Also, 5 injections of heated trypsin (110°C during 30 min) did not have such an effect.

(2) Three axons were injected with trypsin and 3 min later they were placed in cold sea water (2°C) for 2 hr. It was found that all of them had resting and action potentials of the expected normal magnitude 5 min after the temperature was restored to around 22°C (resting potential ranged from 48 to 54 mV and action potential ranged from 85 to 93 mV). Thereafter, the usual set of events previously described was observed. This indicates that the process by which proteases affect

membrane potentials, resistance, and permeability is due to an enzymatic reaction, probably enzymatic hydrolysis of protein.

Discussion.—The experiments have clearly shown that proteases affect membrane potentials, resistance, and permeability, thus indicating that the protein in the inner surface is available to proteolytic action in contrast to the protein of the outer surface.

Before any interpretation the following have to be considered with caution:

(1) If the inner membrane protein is reached by proteases with considerable differences in time (of the order of minutes) and if the physiological membrane as a consequence of this is being destroyed in different regions, the still-functioning adjacent membrane should be affected by leak currents and by the presence of calcium ion leaking in (ref. 11). Here both resting and action potential should approach zero. Membrane resistance should also be reduced. In this case no conclusion can be drawn about the participation of membrane protein in the genesis of the action potential.

(2) On the other hand, if the inner surface is reached in every region of the axon at the same time, the experiment would be conclusive indicating that protein does play an important part in impulse generation as by modifying cell surface phospholipids, perhaps in orientation, packing, or structure to permit the ion fluxes of activity. One wonders about a contractile protein associated with the membrane and capable of deforming it, since giant axons have been shown to shorten reversibly with activity.[12]

Intracellular perfusions of the giant axon *Dosidicus gigas* with trypsin have clarified some of these questions since here the protein access in the membrane is much easier and presumably the whole membrane is under simultaneous effect of proteases.[13]

The experiments reported represent a step toward producing predetermined changes in the giant axon excitable membrane which it is hoped can be correlated with functional consequences.

Summary.—Since external application of proteases seems not to interfere with electrical function of squid, lobster, or frog axons, participation of protein in membrane physiology has been thought by some to be doubtful. The following findings indicate that the protein in the inner surface of the excitable membrane of an axon is available to proteolytic action in contrast to the protein of the outer surface:

(1) Intracellular microinjection of protease affects both resting potential and action potential. The temporal sequence of protease-induced changes suggests that protein is somehow involved in impulse generation.

(2) Intracellular microinjection of protease affects membrane resistance. There is a considerable decrease in resistance to hyperpolarizing currents.

(3) Intracellular microinjection of protease increases both sodium and potassium effluxes.

There is an excellent agreement and consistency between the temporal sequences of protease-induced changes determined by measuring membrane potentials, membrane resistance, and membrane permeability. The present communication, therefore, demonstrates that cell surface protein integrity is essential for maintenance of cell membrane properties and physiology.

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[1] Balantine, R., and A. K. Parpart, *J. Cell. Comp. Physiol.*, **16**, 49 (1940).
[2] Tobias, J. M., *J. Cell. Comp. Physiol.*, **46**, 183 (1955).
[3] Narahashi, T., personal communication (1963).
[4] Nelson, P. G., *J. Cell. Comp. Physiol.*, **52**, 127 (1958).
[5] Tobias, J. M., *J. Cell. Comp. Physiol.*, **52**, 89 (1958).
[6] Tobias, J. M., *J. Gen. Physiol.*, **43**, Suppl., 57 (1960).
[7] Northrop, J. H., *J. Gen. Physiol.*, **9**, 497 (1926).
[8] Rojas, E., and M. Luxoro, *Nature*, **199**, 78 (1963).
[9] Tobias, J. M., *Nature*, **203**, 13 (1964).
[10] Oikawa, T., C. S. Spyropoulos, I. Tasaki, and T. Teorell, *Acta Physiol. Scand.*, **52**, 195 (1961).
[11] Hodgkin, A. L., and R. D. Keynes, *J. Physiol.*, **131**, 592 (1956).
[12] Bryant, S. H., and J. M. Tobias, *J. Cell. Comp. Physiol.*, **46**, 71 (1955).
[13] Tasaki, I., and M. Luxoro, personal communication (1964).

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*ADAPTIVE VARIATION IN THE BREEDING REGIME OF A TROPICAL SEA BIRD**

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The sooty tern (*Sterna fuscata*) is one of the most widely distributed of tropical sea birds, breeding on islands round the world between about 28°N and 31°S.[1] Within this range, different populations of the species show remarkable differences in breeding periodicity, demonstrating unusually clearly the potential evolutionary flexibility of this character, in spite of the apparent adherence by the vast majority of birds to annual regimes under rigid proximate[2] control.

Most populations of sooty terns breed at annual intervals, and in areas where sea-surface temperatures are above 23°C for only part of the year, breeding occurs during this period.[1] However, on Ascension Island in the equatorial Atlantic a population of sooty terns returns to breed at intervals of 9–10 months.[3–5] This was for a time the only proved example of a population of birds which had sexual cycles (defined here as the whole series of events occurring between the production of a fertile egg in two successive breeding periods) with a periodicity independent of any annual variation in the environment: recently it has become clear that in several other tropical sea birds members of certain populations breed at intervals of less than a year, and in different seasons in successive years.[6–8]

It has long been known, however, that on certain islands in the Pacific sooty terns have yet another breeding regime, the colonies being active at two distinct seasons in each year, roughly six months apart. It was at first suggested, by Hutchinson[9] and Chapin,[4] that on such islands there might be two separate populations of sooty terns, each breeding at annual intervals, but at opposite seasons—a situation which has since been found to occur in two forms of the petrel *Pterodroma*