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PROTEIN RELEASE FROM THE INTERNAL SURFACE OF THE SQUID GIANT AXON MEMBRANE DURING EXCITATION AND POTASSIUM DEPOLARIZATION

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Summary

The proteins in the perfusate collected from intracellularly perfused squid giant axons were analyzed after being labeled with radioactive ^{125}I-labeled Bolton-Hunter reagent. The rate of protein release into the perfusate was found to be increased by the following electrophysiological manipulations of the axons: (1) repetitive electrical stimulation at 60 Hz in axons perfused with normal potassium fluoride-containing solution or at 0.125 Hz in axons perfused with tetraethylammonium containing solution, (2) perfusion with 4-aminopyridine solution which induces spontaneous electrical activity in the axon, and (3) depolarization of the axon induced by raising the external potassium concentration. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the proteins released under these conditions yielded molecular weight profiles different from those of the extruded axoplasmic proteins. These observations indicate that there exists, in close association with the axonal membrane, a particular group of proteins, the solubility of which is readily affected by changes in the state of the membrane.

Introduction

Proteins located near the inner surface of the squid giant axon membrane appear to play important roles in the maintenance of membrane excitability. The most direct evidence for this is derived from experiments in which intracellular perfusion of squid giant axons with proteolytic enzymes was found to produce changes in the amplitude and shape of the action potential [1—5]. The nature and role of these proteins in the process of excitation, however, is not yet altogether clear. Using various covalent labeling techniques, we have attempted to elucidate the properties of the proteins on the inner surface of the axon membrane which are related to membrane excitability.

Several previous studies [6—10] showed that the rate of protein release into the perfusate of an internally perfused squid giant axon could be increased as a result of either potassium depolarization of the membrane or intracellular perfusion with chaotropic anions. Takenaka et al. [9] reported differences in membrane associated proteins between stimulated axons and nonstimulated axons. However, the labeling techniques used in all the above studies were not sensitive enough to enable us to characterize fully the properties of the proteins which were released. More recently, we employed ^{125}I-labeled Bolton-Hunter reagent as a covalent labeling probe with a high specific activity (>1500 Ci/mol) with greater success [11]. This reagent reacts with accessible amino groups of proteins [12]. By using this improved technique, we have been able to extend our observations to include molecular weight analyses of protein samples collected from the internal perfusates of squid giant axons subjected to a variety of electrophysiological manipulations.

Methods

Experiments were performed at the Marine Biological Laboratory, Woods Hole, Mass., on freshly excised giant axons of squid (*Loligo pealii*). The technique for the intracellular perfusion employed was that developed by Tasaki et al. [3]. Pronase (Calbiochem) was used to remove the major portion of the axoplasm from the axon interior. This was accomplished by dissolving 0.05 mg pronase in 1 ml of standard internal perfusion solution and passing this solution through the axon interior for 10 min. This mild pronase treatment had no detectable electrophysiological effects. The enzyme was washed out of the axon with an enzyme-free perfusion solution for at least 40 min before the perfusate was collected for analysis. The composition of internal perfusion solution was 400 mequiv. K^+/l, 360 mequiv. F^-/l, phosphate (potassium salt) buffer at pH 7.3 and glycerol (to adjust the osmolarity). The composition of the artificial sea water used was 450 mM NaCl, 10 mM KCl, 50 mM $MgCl_2$, 10 mM $CaCl_2$ and 10 mM tris(hydroxymethyl)aminomethane; the pH was adjusted to 8.0—8.2. The action potential amplitude, the rate of potential rise and the conduction velocity were monitored throughout the course of these experiments.

The proteins in the perfusate were labeled using a method [11] similar to that originally proposed by Bolton and Hunter [12]. Radioactive iodinated *p*-hydroxyphenylpropionic acid *N*-hydroxysuccinimide ester (^{125}I-labeled Bolton-Hunter reagent) was obtained from New England Nuclear at a specific activity of 1500 Ci/mmol. After labeling, the proteins were precipitated with trichloroacetic acid, washed three times with a 1 : 1 mixture of alcohol and ether then once with ether alone and finally dried. This procedure removed the unreacted Bolton-Hunter reagent, labeled lipids and the residual trichloroacetic acid. The radioactivity of the labeled samples was measured with a Beckman Biogamma counter. The proteins were then solubilized in 2.5% Na_2CO_3 and 2% sodium dodecyl sulfate (SDS), and were analyzed by SDS polyacrylamide gel electrophoresis in 7.5% polyacrylamide gels according to the method of Neville [13]. The molecular weight distribution of the proteins on the gel was calibrated using purified proteins of known molecular weights (bovine serum albu-

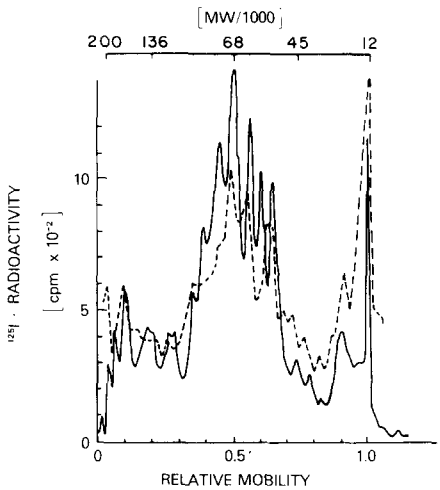


Fig. 1. Molecular weight distribution of proteins in extruded axoplasm. The axoplasmic proteins were labeled with ^{125}I-labeled Bolton-Hunter reagent and separated by polyacrylamide SDS gel electrophoresis. The solid line was obtained by staining the gel with Coomassie blue and scanning at 560 nm. The dotted line shows the radioactivity pattern obtained from the same gel. Abscissa: protein mobility relative to cytochrome *c* (lower) or molecular weight determined with standard proteins (top).

min, 68 000; dimer of bovine serum albumin, 136 000; ovalbumin, 45 000 and cytochrome *c*, 12 000). The gels were sliced into 1.2-mm thick pieces using a Canalco gel slicer and the radioactivity of each slice was counted with a Beckman Biogamma counter.

In order to demonstrate the reliability of this method of labeling, the gel electrophoresis pattern of radioactively labeled proteins in the extruded axoplasm was compared with that of the same proteins stained with Coomassie blue. The densitometric scan (at 560 nm) of the Coomassie blue-stained gel is shown in Fig. 1 (solid line). The gel was then sliced and the radioactivity of each slice was measured. The distribution pattern of the radioactivity obtained is shown as the dotted line in Fig. 1. Comparison of these two patterns shows that there is a good qualitative agreement between the pattern of proteins detected by the Coomassie blue staining and that obtained with the ^{125}I-labeled Bolton-Hunter reagent technique. The apparent quantitative differences, particularly in the low molecular weight regions of the gel (i.e., <45 000) probably reflect the inadequacy of Coomassie blue as a stain for proteins in this range.

Results

Repetitive electrical stimulation

Repetitive electrical stimulation of an axon internally perfused with KF perfusion solution caused a large increase in the quantity of proteins released into the perfusate (Fig. 2). The stimulus frequencies used in these experiments ranged from 60 to 200 Hz. During such high frequency stimulation, the action potential amplitude often declined gradually from approx. 120 mV to 90–100

mV. In the absence of repetitive stimulation the rate of protein release into the perfusate fell back roughly to the original level.

The proteins in the perfusate collected before, during and after stimulation were analyzed by SDS polyacrylamide gel electrophoresis. Molecular weight profiles obtained are shown in Fig. 3. The distribution of radioactivity peaks of the gel differed from that of extruded axoplasm (Fig. 1). The most significant difference was the relatively greater amount of 45 000- and 12 000-dalton proteins present in the perfusate from the stimulated axon. The perfusate samples collected from the axon in the absence of stimulation (control) contained a relatively small amount of proteins at 45 000 and 12 000 daltons (Fig. 3).

Electrical stimulation in tetraethylammonium-treated axons

Intracellular application of tetraethylammonium has been shown to prolong the action potential [15]. Assuming that the conformation of membrane proteins remains altered during prolonged action potentials, we expect to find a similar set of proteins released with low frequency (0.059—0.1 Hz) stimuli in the presence of tetraethylammonium as with higher frequency stimuli in the absence of tetraethylammonium. Fig. 4 shows that the low frequency stimuli in the presence of tetraethylammonium increased the rate of protein release, as expected. In these experiments, the axoplasm was removed with a solution containing 5 mM tetraethylammonium and 90 mM KF for 30 min before the collection of perfusate started. The action potential duration under these con-

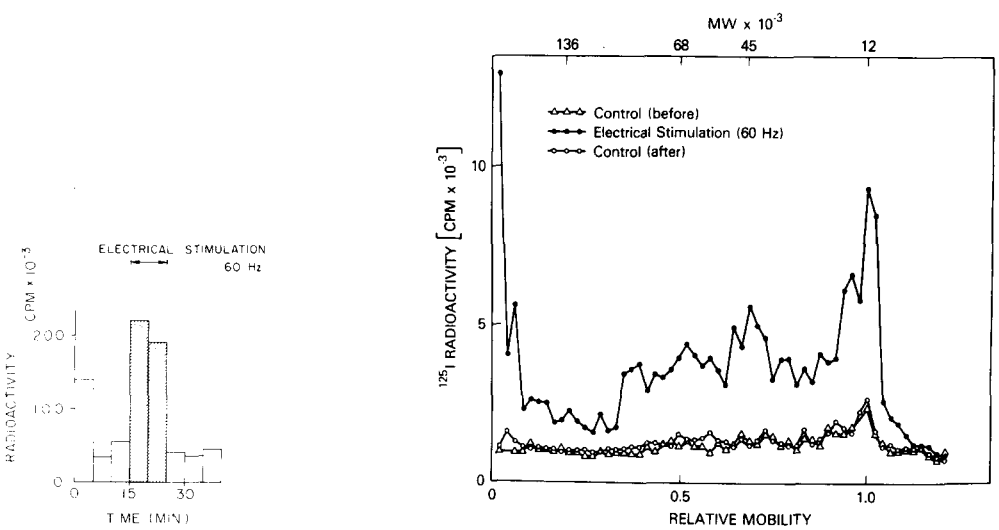


Fig. 2. Effect of electrical stimulation on the rate of protein release into the perfusate. The intracellular perfusion solution contained 90 mM KF and 10 mM potassium phosphate. The stimulus frequency was 60 Hz. The proteins in the perfusate were labeled with ^{125}I-labeled Bolton-Hunter reagent as described in the text.

Fig. 3. Molecular weight distribution patterns obtained by SDS polyacrylamide gel electrophoresis of the labeled proteins in the perfusate collected during repetitive electrical stimulation and in the absence of stimulation.

ditions was 1—3 s and the amplitude was usually decreased from 110 to 80 mV.

The molecular weight distribution of released proteins in the perfusates is shown in Fig. 5. In the control samples (before and after stimulation) the amount of protein was small and no protein peak, except for one at $M_r = 12 \cdot 10^3$, was found. The protein distribution in the perfusate from tetraethylammonium-treated axons during stimulation is quite similar to the molecular weight distribution of the protein released during repetitive stimulation (see Fig. 3), again showing a relative dominance of the $12 \cdot 10^3$- and $45 \cdot 10^3$-dalton peaks.

Intracellular perfusion with 4-aminopyridine

It has been reported that 4-aminopyridine selectively blocks the potassium current and produces spontaneous repetitive firing of action potentials in the squid giant axon [16]. We measured the amount of protein released into perfusate in the presence of this reagent (1—3 mM) and found, in some cases, an increase in the rate of the protein release during the spontaneous firing of action potentials (Fig. 6). The repetitive firing started immediately after application of 4-aminopyridine. The shape and the interval of the action potential were extremely variable, thereby possibly explaining the failure for not observing an increase in protein release in all cases. After replacing the internal solution with the normal perfusion solution free of 4-aminopyridine, the action potential amplitude recovered almost 90%; but the rate of protein release remained high during the following 5 min before declining to control levels (Fig. 6).

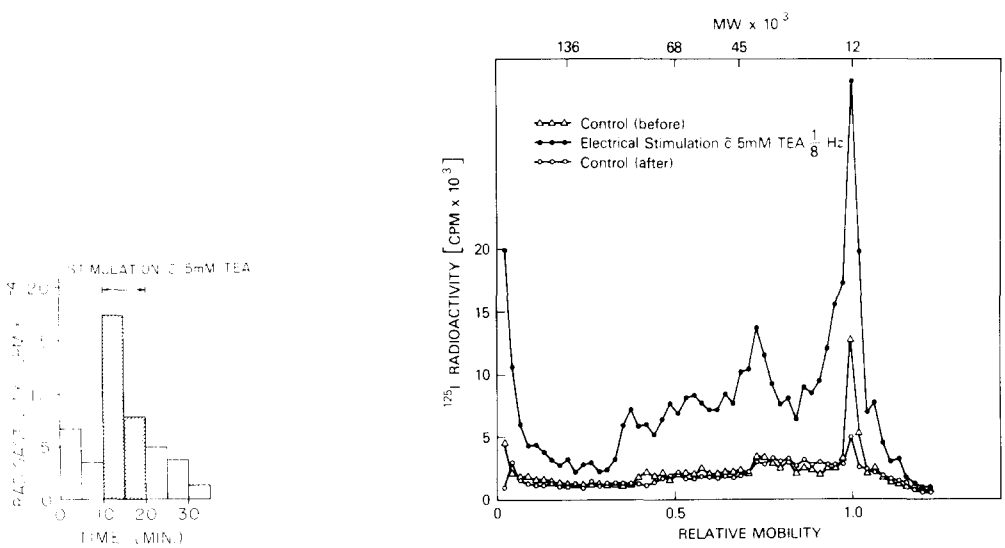


Fig. 4. Effect of electrical stimulation in the presence of 5 mM tetraethylammonium (TEA) internally on the rate of proten release from the axon interior. During 5 mM tetraethylammonium perfusion the action potential duration was 2.6 s. Stimuli were delivered at 7-s intervals. The axon was immersed in artificial sea water in which Mg^{2+} was replaced by Ca^{2+}.

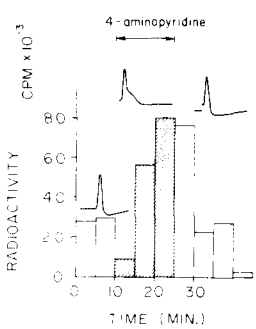
Fig. 5. Molecular weight distribution obtained by SDS polyacrylamide gel electrophoresis of the labeled proteins in the perfusates collected at rest, during repetitive stimulation and at rest. The axon used was internally perfused with a tetraethylammonium-containing solution as in the preceding experiment.

The proteins collected during 4-aminopyridine perfusion were analyzed by SDS polyacrylamide gel electrophoresis. The molecular weight profiles obtained are shown in Fig. 7. The protein profile appeared to be similar to that of the proteins released during electrical stimulation, again indicating a dominace of release of low molecular weight proteins.

Potassium depolarization of the axonal membrane

As long as an internally perfused axon was immersed in artificial sea water, the quantity of protein found in the perfusate was low, but was still detectable, even after an extended period of internal perfusion. When the aritifical sea water was switched to a potassium-rich solution, the quantity of protein in the perfusate was found, initially, to increase dramatically (Fig. 8) and then to decline gradually. Switching back to the normal artificial sea water caused the rate of protein release into the perfusate to fall back roughly to the original level. These observations are consistent with previous findings [6—9].

The proteins which appeared in the perfusate were analyzed by SDS polyacrylamide gel electrophoresis. Fig. 9 illustrates the molecular weight pattern of proteins released by K^+ depolarization. This pattern appears generally comparable to that seen with extruded axoplasm (Fig. 1). The one clear difference from the axoplasm profile is the relatively greater amount of 12 000-dalton protein in the perfusate. This finding of a selective release of 12 000-dalton protein during K^+ depolarization is in agreement with previous reports [6—9], where different covalent labeling probes were used. However, in those studies



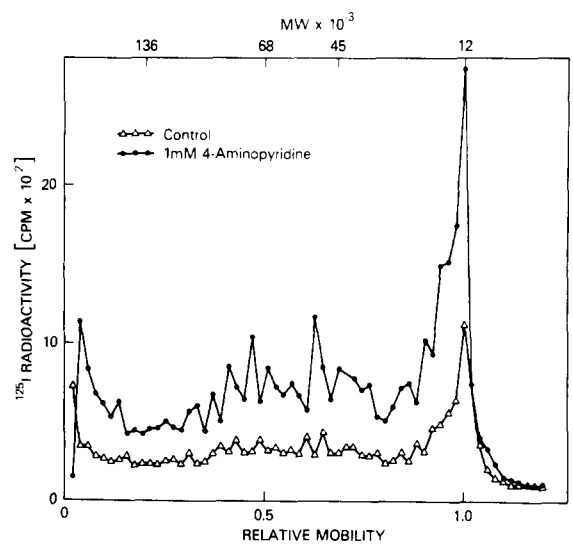


Fig. 6. Effect of 4-aminopyridine on the rate of protein release into the perfusate. The right- and left-hand traces of action potentials in the figure were evoked by external stimulation: the action potential in the middle was triggered by internal perfusion with a solution containing 4-aminopyridine.

Fig. 7. Molecular weight distribution patterns obtained by SDS gel electrophoresis of ^{125}I-labeled Bolton-Hunter reagent labeled proteins in perfusate collected before (control) and during internal perfusion with 4-aminopyridine.

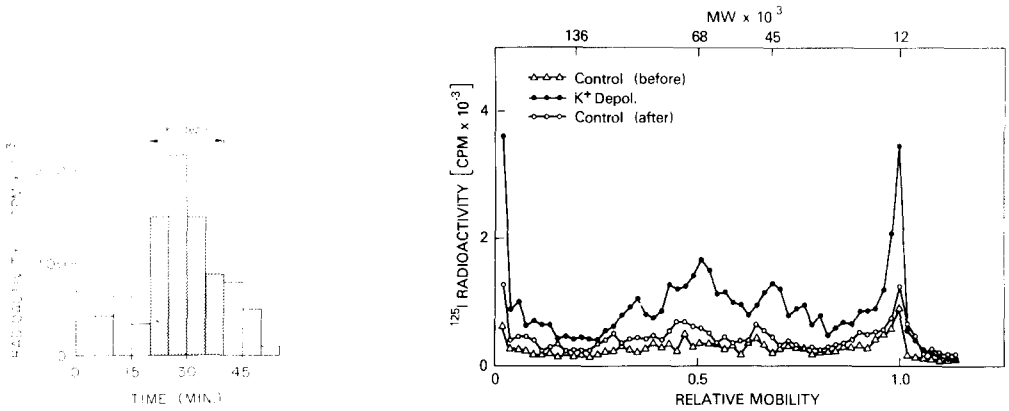


Fig. 8. Release of proteins into perfusate at rest and during potassium depolarization. The depolarization was produced by switching the external artificial sea water to a potassium-rich medium. The salt composition of the potassium-rich solution was 250 mM NaCl, 200 mM KCl, 60 mM $CaCl_2$ and 10 mM Tris (the pH of the external solution was adjusted to 8.1).

Fig. 9. Molecular weight distribution patterns obtained by SDS polyacrylamide gel electrophoresis of the proteins in the perfusates collected in the normal and depolarized states. The depolarization was produced as described in the legend to Fig. 8. K^+-Depol, potassium depolarization.

the higher molecular weight components also released into the perfusate were not resolved. In contrast to the previous three stimulation paradigms (Figs. 3, 5, 7), the release of the 45 000-dalton protein does not appear to be selectively enhanced by potassium depolarization.

Discussion

We have shown that the amount of protein released into the perfusate of an internally perfused squid giant axon increases as a result of a variety of manipulations which affect membrane excitability. Analysis by the method of SDS polyacrylamide gel electrophoresis revealed that the protein distribution in the perfusate from electrically stimulated axons differs from that of extruded axoplasm.

The pronase pretreatment of the axons removes the bulk of the axoplasm [2], but leaves a thin protein layer beneath the membrane. This thin layer has been visualized by scanning electron microscopy as a three-dimensional network structure of filamentous proteins [14]. The thickness of the layer is estimated to be of the order of 1 μm. We presume that the protein released during excitation originates from this layer.

Two major differences are seen between the axoplasmic proteins (Fig. 1) and the proteins from samples of perfusate collected during stimulation (Figs. 3, 5, 7, 9). First, in the perfusate samples the peak in the 12 000-dalton region is considerably higher than the peak in 68 000-dalton region, whereas in the axoplasm these two peaks are of comparable height. Second, in all of the perfusate samples, except those from the potassium depolarized axon, the peak in the 45 000-dalton region is higher than that in the 68 000-dalton region, whereas the opposite is true in the sample from axoplasm. However, the existence of a

large amount of high molecular weight (>200 000) proteins in the perfusate samples collected during stimulation is somewhat uncertain, since we used 7.5% polyacrylamide gels which have a poor resolution in this molecular weight range. Further analyses using less concentrated acrylamide gels are necessary to clarify this issue.

The preferential release of the 12 000 and 45 000 molecular weight proteins is interesting since (1) the molecular weight of the calcium binding protein isolated from the axoplasm of squid giant axons by Alema et al. [17] is very close to 12 000 daltons, and (2) actin (M_r = 46 000) has been found to be a submenbranous protein in several organisms [18]. It will be important in future experiments to determine whether these specific proteins are selectively released into the perfusate by electrical stimulation, and, if so, what role they play in the excitation process. The dominance of the 68 000-dalton protein in the perfusate seems to be characteristic of potassium depolarization. Furthermore, this protein is also released by internal application of chaotropic anions (Pant, H.C. and Terakawa, S., unpublished). This difference in molecular weight profiles from the stimulation paradigms suggests that potassium depolarization disturbs the submembrane structure more profoundly than does excitation.

The mechanisms of release of submembrane proteins during excitation are unclear at present. One possible explanation is that the proteins in the submembranous layer correspond to the peripheral proteins of the axonal membrane as described by Singer and Nicholson [19], and that these proteins are solubilized during electrophysiological manipulations of the axons described in this paper. Since the loss of these proteins leads to suppression of excitability [8], they could be regarded as a part of the excitable membrane. During nerve excitation or potassium depolarization, the altered ionic environment may induce changes in tertiary and quaternary structure of the macromolecules, either by virtue of specific ionic effects or by general salt effects [20], so as to increase their solubility in the perfusing KF solution. Alternatively, there may be a dynamic interaction between intrinsic proteins in the membrane and the ectoplasmic proteins, as has been shown for erythrocytes [21] and lymphocytes [22]. The conformational change, whatever the cause may be, taking place in the intrinsic proteins could disrupt the interaction and liberate the submembranous proteins.

The existence of extensive conformational changes of the protein near the membrane associated with nerve excitation is already known through optical studies of squid axons [23,24]. These observations and the present findings indicate that, in intact axons, specific groups of proteins attached to the membrane may be loosened by membrane excitation. The optical changes observed during nerve excitation, as well as physicochemical changes of the membrane proteins described in this paper, seem to indicate that there is transmission of some information from the axolemma to the protein layer beneath the axolemma.

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