Release of Proteins from the Inner Surface of Squid Axon Membrane Labeled with Tritiated N-Ethylmaleimide

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ABSTRACT Proteins in the inner surface of the squid axon membrane were labeled by intracellular perfusion of [3H]N-ethylmaleimide (NEM), which forms covalent bonds with free sulfhydryl groups. The excitability of the axon was unaffected by the [3H]NEM perfusion. After washout of the unbound label, the perfusate was monitored for the release of labeled proteins. Labeled proteins were released from the inner membrane surface by potassium depolarization of the axon only in the presence of external calcium ions. Replacement of the fluoride ion in the perfusion medium by various anions also caused labeled protein release. The order of effectiveness was SCN- >> Br- > Cl- > F-. The extent of labeled protein release by the various anions was correlated with their effects on axonal excitability. The significance of these results is discussed.

INTRODUCTION

Squid giant axons are known to maintain their electrical excitability after extensive removal of the axoplasm with proteases (Takenaka et al., 1968). Prolonged treatment of the axon membrane with intracellularly administered pronase initially produces a decrease in the maximum inward current under voltage clamp (Sato et al., 1973), before the decrease in amplitude and prolongation of the duration of the action potential is observed (Takenaka and Yamashigi, 1966; Armstrong et al., 1973). Ultimately, the proteolytic action causes inexcitability of the axon (Tasaki, 1968). Such observations suggest that proteins associated with the internal surface of the axon membrane are involved in the regulation of excitability.

In an earlier investigation (Gainer et al., 1974), the proteins in the internal membrane surface of the squid axon were labeled with 125I by using an enzymatic radio-iodination procedure in combination with the intracellular perfusion technique. This study demonstrated that a protein with a molecular weight equal to about 12,000 daltons was associated with the internal membrane surface, and the ability of this protein to be iodinated was selectively reduced by potassium depolarization of the axon. Recent studies by Takenaka et al. (1976) have con-
firmed these findings, and suggest that the labeled 12,000-dalton protein is released into the perfusate after potassium depolarization or electrical activity of the axon. In the present study, we have labeled the proteins in the membrane by intracellular perfusion of [3H]N-ethylmaleimide ([3H]NEM) and have monitored the perfusate for release of labeled proteins after various experimental treatments of the axon. We report here that labeled proteins are released from the internal surface of the membrane by potassium depolarization (in the presence of external calcium ions only), and by the internal perfusion of various anions.

MATERIALS AND METHODS

Electrophysiological Methods

Giant axons of squid Loligo pealei available at the Marine Biological Laboratory, Woods Hole, Mass., were used throughout the present experiments. The diameter of the axons used was between 280 and 380 μm. The major portion of small nerve fibers and other adherent tissues surrounding the giant fiber were removed under a dissecting microscope. The axon was then transferred to a Lucite chamber filled with artificial seawater (ASW) in which internal perfusion with two glass cannulae was performed (Lerman et al., 1969). The outside diameter of the inlet cannula was 120 μm (see Fig. 1, right). The standard internal perfusion solution contained 400 meq/liter K-ion, 350 meq/liter F-ion, phosphate (potassium salt) buffer adjusted to a pH between 7.2 and 7.4, and glycerol (4% by volume). In the present study the axoplasm within a long portion of the axon, 28-30 mm in length, was removed extensively by means of enzymatic digestion. Axons used were initially perfused intracellularly with the KF solution containing 0.01 mg/ml pronase (Calbiochem, San Diego, Calif.) until the maximum inward current under voltage clamp was reduced to about 80% of the level before the start of internal perfusion (approximately 12-20 min). After replacement of the enzyme-containing solution with an enzyme-free one, the perfusion zone was shortened by inserting both of the cannulae 5 mm deeper into the axon so that the axoplasm remaining in the unperfused zone was separated from the perfusion zone by the nonflowing KF solution. The length of the perfusion zone was thus between 18 and 20 mm. The flow rate of the internal perfusion fluid was maintained at a level between 20 and 35 μl/min.

To study the effects of internal anions, the KF in the standard perfusion solution described above was replaced by an equimolar amount of KCl, KBr, or KSCN. The composition of the ASW used was 423 mM NaCl, 9 mM KCl, 25 mM MgCl₂, 25 MgSO₄, 10 mM CaCl₂, and 5 mM Tris (the pH of the external solution was adjusted to between 7.8 and 8.0). The composition of the external potassium depolarization solution was identical to the above ASW except that the NaCl was reduced to 250 mM and the KCl raised to 200 mM. When Ca-ions were removed from the K-rich ASW, ethylene glycol bis (β-aminoethyl ether) tetraacetic acid (EGTA) was added to a concentration of 4 mM. In all cases the osmotic pressures of the various external solutions were equated by the addition of small amounts of glycerol, and the pH was between 7.8 and 8.0.

The potential difference across the membrane was measured at the center of the perfusion zone with a glass pipette Ag-AgCl electrode filled with a 3 M KCl solution (70 μm in diameter). The pipette was filled with KCl-agar gel near the tip to reduce outflow of the KCl solution. A calomel electrode immersed in the external medium was used as the reference point for potential measurements. The external fluid medium was grounded through a large coil of platinized platinum wire. Stimulating shocks were delivered through a pair of platinum electrodes to the unperfused zone of the axon when
propagation of impulses across the perfusion zone was monitored. When application of either current pulses or voltage pulses to the membrane was required, a platinized platinum wire (50 μm in diameter) was inserted into the axon through the outlet cannula.

Biocelhemical Methods

Tritiated N-ethylmaleimide ([3H]NEM, sp act 230 mCi/mmol) was purchased from New England Nuclear, Boston, Mass. The [3H]NEM (250 μCi) was dissolved in 20 ml of the 400 mM KF solution. The NEM concentration in this solution was approximately 50 μM. The [3H]NEM-containing KF solution was introduced into the axon from which the axoplasm had been removed by the method described above, and the axon was perfused with this solution for 15 min. During this period, the axon was immersed in rapidly circulating (50 ml/min) artificial seawater. No detectable deterioration of the membrane excitability was produced by the [3H]NEM treatment under the present experimental conditions. After switching to a [3H]NEM-free KF solution, continuous flow of both the internal and external fluid media was maintained for 15-20 min in order to eliminate free [3H]NEM from this system. The radioactivity of the total proteins that could be extracted from one whole single fiber preparation was of the order of 10^4 cpm. (A small amount of efflux of [3H]NEM across the membrane [20-30 cpm/s·cm^2] was observed during internal application of the [3H]NEM-containing KF solution.) Although NEM is commonly considered specific for sulfhydryl groups, it can also react to some extent with amino groups. At the pH of the internal perfusion fluid, the reaction rate of NEM with thiols is about 1,000-fold greater than with amino compounds (Means and Feeny, 1971). Therefore, under these conditions, it is likely that the principal reaction of the [3H]NEM in this study was with the free sulfhydryl groups in membrane proteins.

The labeled proteins released from the membrane into the internal perfusing medium were measured by collecting fractions of perfusate from the outlet cannula onto pads of Whatman 3 mm filter paper (Fig. 1). Contamination by free [3H]NEM in each collected
sample was eliminated by precipitating proteins with trichloroacetic acid (TCA). The TCA precipitation procedure consisted of two washes of the pads in ice-cold 10% TCA (30 min each), followed by a hot (95°C) extraction in 5% TCA (30 min), another 30 min in cold 5% TCA, a 30-min wash of the pads in ethanol:ether (1:1, vol/vol), and then 30 min in 100% ether. In control experiments it was demonstrated that any protein-bound \[^{3}H\]NEM and not free \[^{3}H\]NEM remained in the pad after this procedure. The radioactivity of the proteins remaining on the filter paper was measured with a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

In one series of experiments perfusate was collected in small glass test tubes. Standard proteins (bovine serum albumin, 68,000 daltons; ovalbumin, 45,000 daltons; and cytochrome c, 12,000 daltons) were added as carrier, and the proteins in the tubes were precipitated with TCA. The pellet was washed with ether to remove the TCA, and then dissolved in 1% SDS, and reduced by the addition of β-mercaptoethanol. The proteins in these samples were then separated by polyacrylamide gel disc electrophoresis in SDS (Neville, 1971). The standard proteins in the coprecipitate also served as internal molecular weight markers on each gel. The gels were sliced, liquid scintillation cocktail was added, and the radioactivity of each slice was measured with a Beckman liquid scintillation counter. All the experiments were conducted at room temperature (20° ± 1°C).

**RESULTS**

**Induction of Protein Release from Membrane by Potassium Depolarization**

After the labeling of the squid axons with \[^{3}H\]NEM and the washout of the unbound label (see Materials and Methods), the axons were continuously perfused with the standard 400 mM KF solution in circulating ASW. Under these conditions the axons maintain their ability to propagate nerve impulses across the perfusion zone for more than 2 h. The resting potential of the exitable membrane was between −55 and −60 mV, and the membrane resistance was usually between 0.7 and 1.5 kΩ·cm². The external ASW was then switched to a K-rich solution, which depolarized the axon by approximately 50 mV. The membrane resistance of the depolarized axon was about 200 Ω·cm².

The effect of such membrane depolarization on the release of proteins into the internal fluid medium was examined. The experimental procedure is schematically illustrated on the right side of Fig. 1. Perfusate was collected at 3-min intervals, and the radioactivity of proteins in each collected sample was measured. A typical example of the results obtained is represented on the left side of the figure. The abscissa indicates the time in minutes after the start of the collection of perfusate. The rate of release of proteins (cpm/3 min) was maintained at a low level as long as the axon was immersed in ASW. After replacement of the external seawater with the K-rich solution, there was a significant rise in the rate of protein release followed by a gradual decline. Replacement of the K-rich external solution by seawater outside the axon brought about a rapid fall in the rate of protein release which finally returned to the original low level. In general, both the membrane potential and membrane resistance gradually approached the original values of the resting state after recirculation of seawater. Conduction could usually be restored during repolarization of the membrane.

In a separate experiment, the radioactive proteins which appeared in the
perfusion were analyzed by SDS gel electrophoresis. In Fig. 2 the broken line shows the molecular weight distribution of proteins released into the internal fluid medium before depolarization, and the solid line shows the molecular weight distribution of proteins released during depolarization. The depolarization was produced by immersing the axon in the K-rich solution. One distinct peak with approximately 12,000 daltons was produced by the potassium depolarization. These results are consistent with those reported in previous papers (Gainer et al., 1974; Takenaka et al., 1976) in which membrane labeling was done by radio-iodination.

**Ca**<sup>++</sup>-Dependence of Protein Release

The data in Fig. 3 illustrate the dependence of protein release upon Ca<sup>++</sup> in the internal medium. Axons were internally perfused with the KF solution. The external solutions used are given in the figure (see Materials and Methods for composition of solutions). As illustrated in Fig. 3 A and B, no significant alteration of the rate of release of radioactive proteins in the internal perfusion fluid could be produced if the membrane was depolarized with the K-EGTA solution. However, as shown in Fig. 3 B, there was sharp rise in the rate of protein release when CaCl₂ was added to the external K-rich medium by replacing the K-EGTA solution with the K-Ca solution. In both of the experiments in Fig. 3, conduction

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**Figure 2.** Molecular weight distribution on SDS polyacrylamide gels of a fraction of [³H]NEM-labeled proteins which were present in the perfusate under normal resting state (NORMAL) and during potassium depolarization (K-DEPO.). The depolarization was produced by replacing the external seawater with the K-rich solution containing CaCl₂. Note that a 12,000-dalton labeled protein was released by potassium depolarization.
of the action potential could be restored by recirculation of seawater outside the axons. The membrane potentials and resistance of the depolarized axon in the K-EGTA and K-Ca++ solutions did not significantly differ.

Variation of the Mg^{2+} concentration of the K-EGTA solution between 5 and 50 mM did not affect the rate of protein release. These findings suggest strongly that the release of proteins during potassium depolarization arises from the interaction between external Ca-ions and the macromolecules at the inner surface of the membrane, presumably due to the enhanced rate of Ca^{2+} entry into the membrane during potassium depolarization (Baker, 1972).

**Figure 3.** Effect of external Ca^{2+}-ion on release of [H]NEM-labeled proteins during potassium depolarization. Two kinds of K-rich solutions containing 200 mM KCl were used to depolarize the membrane. K-EGTA solution contained 4 mM EGTA but no Ca^{2+}-ions. K-Ca solution contained 10 mM CaCl_2, but no EGTA.

**Effects of Internal Anions on Protein Release and Excitability of the Axon**

Previous studies have demonstrated that the excitability of the perfused squid axon is strongly affected by the anionic species used in the perfusion fluid (Tasaki et al., 1965). The efficacy of internal anions in maintaining normal excitability increased in the order of SCN^- < I^- < Br^- < Cl^- < SO_4^{2-}, <HPO_4^{2-}, <F^- This anion sequence corresponds to the lyotropic (or Hofmeister) series and is directly related to the salting-out effect of anions on proteins, where SCN^- is least effective and F^- most effective (see Tasaki et al., 1965, for a discussion of these phenomena in relation to the squid axon).

The following experiments indicate that such anion effects on membrane excitability are correlated with the ability of the anions to release membrane proteins into the internal fluid medium. The [H]NEM-labeled squid axons immersed in ASW were first perfused intracellularly with a 400 mM KF solution, and then with a 400 mM solution of KCl, KBr, or KSCN for a period of 6 min. After these treatments, the KF solution was reintroduced into the axons.

Excitability was suppressed within a short time after replacement of the internal KF solution with other K-anion solutions. (Deterioration of excitability was accompanied by a fall in membrane resistance.) The average survival time after onset of internal perfusion with the KCl solution, as determined by eight
individual measurements, was 3.4 ± 2.4 (SD) min; the average survival time with the KBr solution internally was 2.1 ± 1.7 (SD) min (nine axons). Loss of excitability took place (within 30 s). It should be noted that the average survival time obtained in the present study was almost 10% of that reported by Tasaki et al., (1965). This enhanced effect was solely dependent on the extent of removal of the axoplasm from the axon, and not on the [3H]NEM treatment.

Reintroduction of the KF solution to the axon interior tended to restore membrane excitability previously suppressed by internal treatment with the other K-anion solutions. The degree of recovery was strongly dependent on the anion species in the internal perfusion fluid. To compare the anion effect on the ability of the axon to restore its excitability, attempts were made to measure the membrane current under voltage clamp before and after treatment of the axon with K-salt solutions. The voltage current relationships illustrated in Fig. 4 show such anion effects. Curve a in each diagram represents the control obtained before applying the K-salt solution to the axon interior. Curve b was obtained from the same axon when the maximum recovery of excitability was attained by reintroduction of the KF solution. Fig. 4A was taken from an axon treated internally with the KCl solution. The magnitude of the action potential associated with excitation could be restored almost completely but recovery of the maximum inward current was only 50%. With this degree of recovery, conduction of action potential could be produced by electric stimulations. As shown in Fig. 4B, the degree of recovery was poorer when an axon had been treated intracellularly with the KBr solution instead of with the KCl solution. There was

![Figure 4](image-url)
no complete recovery of the magnitude of the emf jump associated with excitation. The maximum inward current was restored by only 20%. In general, axons lost their ability to propagate action potentials across the perfusion zone under these conditions. As shown in Fig. 4C, the axon was no longer capable of producing inward current in response to depolarizing voltage pulses. No all-or-none action potential could be elicited from the axon in this case. Membrane resistance could be restored only to a level of 20% of that before the KSCN treatment.

A large amount of protein was released into the internal fluid medium during internal perfusion with these K-anion solutions. The amount of protein released was strongly dependent on the anion species in the internal perfusion fluid; SCN ions released about 20%, Br ions released about 6%, and Cl ions released about 3% of the total radioactivity in the squid fiber. Typical examples of the results obtained are shown in Fig. 5. Fig. 5A was obtained from an axon internally perfused with the KCl solution. The rate of release of proteins into the internal fluid medium (cpm/3 min) was enhanced about three times by replacement of the KF solution with the KCl solution. Reintroduction of the KF solution into the axon brought about a fall in the rate of protein release which approached the original low level within 6 min.

Fig. 5B shows that intracellular application of the KBr solution released proteins at a rate approximately six times as high as that under internal perfusion with the KF solution. Fig. 5C shows that introduction of the KSCN solution to the axon interior produced a dramatic rise in the rate of protein release.
followed by a quick fall. In this case, the rate was reduced to a level much lower than the original level by replacement of the internal KSCN solution with the KF solution. Thus, it seems that the major portion of releasable proteins appeared in the perfusate within a short time after onset of internal perfusion with the KSCN solution.

Results similar to those shown in Fig. 5 could be obtained from the [3H]NEM-labeled squid axons immersed in Ca²⁺-free ASW containing EGTA. The release of proteins by internally applied anions does not require the presence of Ca-ion in the external medium.

**D I S C U S S I O N**

Release of proteins from the inner surface of the squid axon plasma membrane was produced either by exposing the external surface of the axon to K⁺-rich solutions or by application of various anions to the internal surface of the axon membrane. The dependence of protein release by K⁺ depolarization upon external calcium ions suggests that this phenomenon is related to an increased calcium concentration at the inner membrane under these conditions.

The empirical application of salt perturbation in protein systems has been widely employed by biochemists. In particular, CaCl₂ and KSCN have been used for protein subunit dissociation (von Hippel and Schleich, 1969). The order of effectiveness of these ions in promoting stable protein conformations (i.e. helical and native conformations, loss of solubility, referred to as “salting-out”) and causing decreased structural stability associated with greater solubility (i.e. random coil structure or denaturation, referred to as “salting-in”) follows the classical Hofmeister series (Bungenberg de Jong, 1949; von Hippel and Schleich, 1969). This ranking corresponds to the order of effectiveness of anions found to release proteins from inner membrane surface (Fig. 5).

Singer and Nicolson (1972) have classified membrane proteins into “ peripheral” (extrinsic) and “integral” (intrinsic) proteins largely on the basis of their solubility characteristics. Integral membrane proteins are hydrophobic and amphipathic, and involved in the structural integrity of membranes; whereas peripheral membrane proteins are considered to be loosely bound to the membrane and not stabilized by hydrophobic interactions. It is of interest to note that it is the latter which are uniquely associated with the cytoplasmic surface of the membrane (Steck, 1974), and that it may be this population of proteins which are being released in our experiments.

As pointed out earlier, it has been demonstrated that ions which tend to solubilize proteins in the squid axon also tend to suppress membrane excitability when applied internally. For example, introduction of Ca-ions into the interior of an axon causes a fall in the membrane resistance and eventual loss of excitability (Tasaki, 1968). The adverse effect of the internal anions on excitability was demonstrated by Tasaki et al. (1965) as well as in the present study (Fig. 4). In contrast, the external surface of the axon membrane is quite insensitive to alterations of the anion composition in the external fluid medium, and calcium in the external medium plays an essential role in stabilizing the membrane macromolecules which are responsible for conformational changes associated with nerve excitation.
Although considerable attention has recently been directed at the subsurface structure beneath the cytoplasmic membrane (Hyden, 1974; Metuzals, 1969; Metuzals and Izzard, 1969; Metuzals and Mushynski, 1974; Le Beux and Willemot, 1975), it is still too early to identify those submembrane structures related to the released proteins in this study. Recently, the fine structure of the pronase-treated squid axon membrane was examined by scanning electron microscopy (Metuzals and Tasaki, 1976). The results obtained clearly indicated the existence of a longitudinally oriented structure made of fibrous material adhering to the inner surface of the membrane. Extensive pronase treatment of the axon interior reduced the amount of fibrous material but did not completely destroy the longitudinally oriented structure. It is therefore plausible that the major portion of the proteins which appeared in the perfusate was released from these fibrous submembrane structures. If that is the case, this submembrane structure may be involved in the regulation of excitability and hence should be regarded as a part of the excitable membrane.

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