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# TETRODOTOXIN AFFECTS SUBMEMBRANOUS CYTOSKELETAL PROTEINS IN PERFUSED SQUID GIANT AXONS

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SUMMARY: Application of tetrodotoxin or saxitoxin to intracellularly perfused squid giant axons caused the release of protein from the cytoskeletal network underlying the axolemma into the stream of perfusing solution. This protein was analyzed by one-dimensional polyacrylamide gel electrophoresis and found to be composed of the following major components: tubulin, actin and proteins having molecular weights of 96, 69 and 38 K-daltons. This observation is consistent with the hypothesis (1,2,8) that the integral membrane proteins controlling excitability in the axolemma (channel proteins) interact with the underlying cytoskeleton to maintain the stability of the excitable membrane.

## INTRODUCTION

There are several lines of evidence which support the hypothesis that the cytoskeletal network forming an undercoating to the axolemma (1-8) is vital in maintaining the excitability of the squid giant axon. This evidence includes: i) the observations that the intracellular perfusion of a squid giant axon with a solution containing a chaotropic anion (e.g. $Br^-$, $I^-$, $SCN^-$) causes prompt loss of excitability simultaneously with the release of these proteins into the perfusion solution (1); ii) electrical stimulation of the axon also causes release of protein from this subaxolemmal layer (2); and iii) drugs (such as colchicine, vinblastine, cytochalasin B and phalloidin) which interact with cytoskeletal proteins have been shown to have electrophysiological effects on voltage clamped

Abbreviations.
TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; TTX, tetrodotoxin; STX, saxitoxin; PAGE, polyacrylamide gel electrophoresis

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axons (7). In the present work, we have attempted to examine further the interactions between excitable sites or channel proteins and the underlying cytoskeleton, by using pharmacological agents known to affect nerve excitability. Tetrodotoxin (TTX) and saxitoxin (STX) are well known neurotoxins which block the action potential and inhibit the inward current of a voltage clamped axon (9,10). By using the same approach used in our previous work (2), we applied these toxins externally to the intracellularly perfused squid giant axon and collected the perfusate, for protein analysis, as it flowed out of the axon. The results of this analysis showed that these toxins cause the release of proteins from the underlying cytoskeleton into the flowing perfusion solution.

## MATERIAL AND METHODS

### Intracellular Perfusion of Squid Giant Axons

Giant axons from the squid (Loligo pealei), available at the Marine Biological Labs, Woods Hole, MA, were used throughout the present investigations. The procedures used for dissection of the giant axons, and for intracellular perfusion have been described previously (2).

### Quantitative Analysis of the Released Proteins

Fractions of perfusate were collected and the proteins in the fractions were labelled with $^{125}$I-Bolton-Hunter reagent (New England Nuclear) as previously described (2). Labelled proteins were separated from unreacted $^{125}$I-Bolton-Hunter reagent by trichloroacetic acid (TCA) precipitation of the protein as previously described (2).

### Qualitative Analysis of the Released Protein

Once the proteins were TCA precipitated, they were very difficult to redissolve, even in the presence of sodium dodecyl sulfate (SDS), $Na_2CO_3$ and β-mercaptoethanol, thus rendering further analysis of these proteins by polyacrylamide gel electrophoresis (PAGE) very difficult. We therefore developed an alternative procedure which avoided TCA precipitation. In this procedure, after labelling the proteins with $^{125}$I-Bolton-Hunter reagent, the proteins were separated from unreacted reagent by gel filtration through a Bio-gel P6 (Bio-Rad) column (1.5 x 5 cm) equilibrated with 100 mM ammonium acetate. The proteins were concentrated by lyophilizing, re-dissolved in a solution of 8 M urea, 2% SDS, 5% β-mercaptoethanol and run on polyacrylamide slab gels (7-11% acrylamide gradient) run according to Neville (11). The labelled proteins were visualized by autoradiography of the dried gel.

In order to insure that none of the proteins being studied were degraded by the pronase used to remove the bulk of the core axoplasm, in these experiments we used trypsin (0.05 mg/ml) rather than pronase: once the core axoplasm was removed, the trypsinization was stopped by adding 0.1 μg/ml soybean trypsin inhibitor to the internal perfusion solution. This trypsin inhibitor was identified as the bottom two bands in the gel shown in Fig. 2.

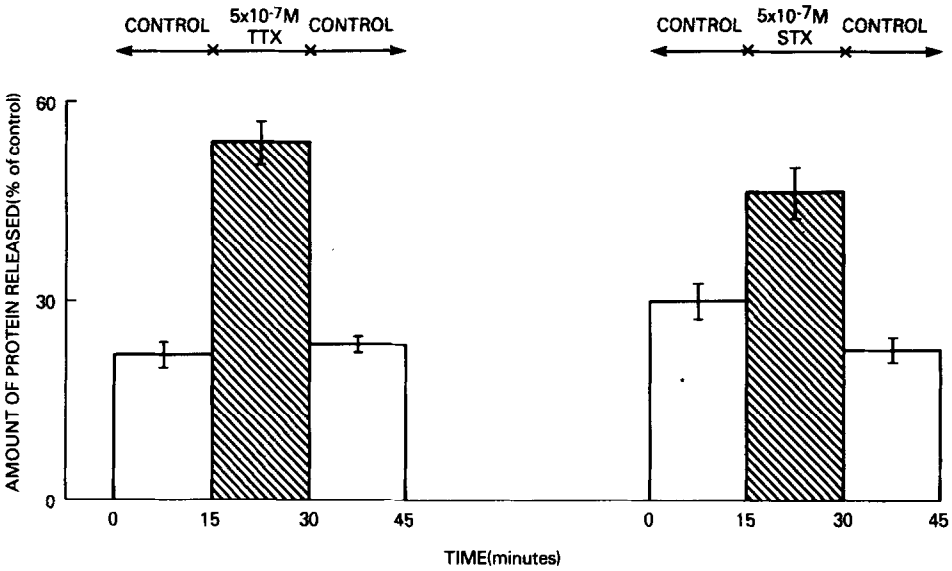


Figure 1. Quantitative analysis of released protein. Fractions of perfusate were collected, labelled with $^{125}$I-Bolton-Hunter reagent, TCA precipitated and counted. Data from 16 axons were averaged in the left-hand graph (for TTX) and data from 5 axons were averaged in the right-hand graph (for STX). The error bars represent the standard error. An analysis of variance indicated a statistically significant difference between the control and TTX-treated sample at the 1% level ($p \leq 0.01$).

## RESULTS

The results of the quantitative analysis of released proteins are shown in Fig. 1. In these experiments, the bulk of the axoplasm was removed by perfusing the axon for 2 min. with a dilute (0.05 mg/ml) solution of pronase. The axon was then perfused for 45 min. with a pronase-free internal perfusion solution to wash out any remaining core axoplasm, degraded proteins, and pronase. The experiments were started at this point by collecting fractions of perfusate. The data in Fig. 1 shows the relative amount of protein released before, during and after exposure of the axon to either 5 x $10^{-7}$ M TTX (left-hand panel) or 5 x $10^{-7}$ M STX (right-hand panel). Since the total amount of protein released varied depending on the size of the axon, the data in Fig. 1 is expressed as relative amounts of protein: that is, the total amount of protein released in the entire 45 minute period shown in the figure was determined, and the amount of protein in each 15 minute period (either control or

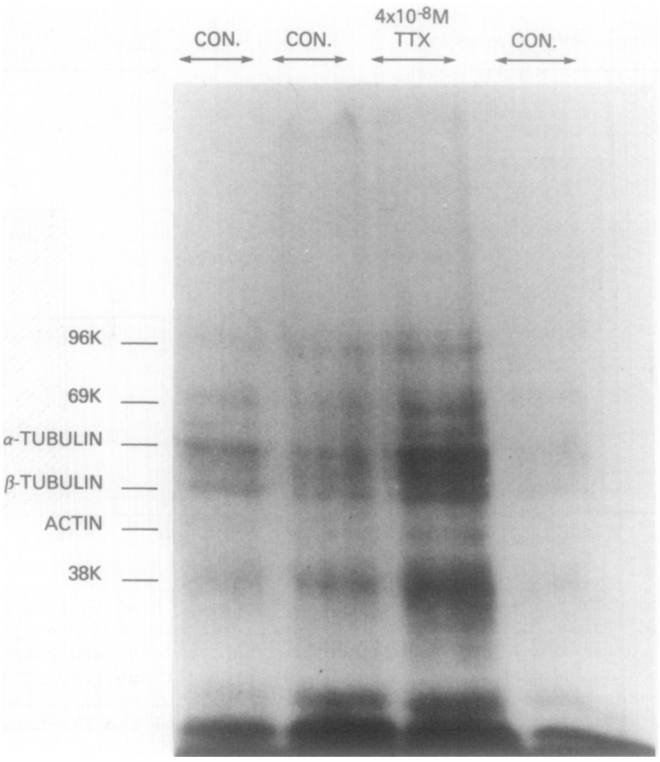


Figure 2. PAGE analysis of released proteins. Fractions of perfusate were collected, labelled with $^{125}$I-Bolton-Hunter reagent, separated on Bio-gel P6 column and analyzed by PAGE. The first two lanes show the proteins being eroded under control conditions, the third lane shows the proteins released during TTX application and the last lane shows the proteins released following TTX application. The bottom two bands in the gel are soybean trypsin inhibitor used to prevent any trypsinization (see Methods).

toxin treated) was expressed as a percent of the total. This data shows that treatment of the axon with TTX or STX caused a significant increase in the amount of released protein. The action potential was blocked by these treatments and could be restored by washing out the toxin. Application of TTX at a concentration as low as 30 nM still caused protein release, although at this concentration there was a longer latency between the application of the toxin and the loss of the action potential. Internal application of TTX had no effect in releasing protein nor did external application of α-bungarotoxin or procaine (only 1 experiment). Mechanical agitation of the axon resulting from changing the external solution was shown to have no effect.

Fig. 2 shows that PAGE analysis of the released protein. The first two lanes of the gel show sequential samples of perfusate before application of TTX. The third lane shows the proteins released during TTX application and the last lane shows the proteins released following TTX application. These gels indicate that the proteins released by TTX are similar, qualitatively, to those in the perfusate under the control conditions. The most prominent proteins in these gels include a 96K protein, a 69K protein, tubulin, actin and a 38K protein and several minor proteins. Actin and tubulin were identified solely by their molecular weights. From previously published (7) two-dimensional PAGE analysis of whole axoplasm, however, it is clear that actin is the principal protein in squid axoplasm with a molecular weight of around 43K. In these experiments, trypsin, rather than pronase, was used to remove the bulk of the axoplasm: all subsequent solutions contained 0.1 μg/ml trypsin inhibitor (bottom two bands on the gels) to prevent further proteolysis.

## DISCUSSION

The results of the present experiments demonstrate that exposing an intracellularly perfused squid giant axon to TTX or STX causes a release of protein into the perfusion solution. Although this quantitative effect is quite pronounced (see Fig. 1), the qualitative analysis of these proteins by PAGE shows that the proteins released by this treatment are essentially the same as those released under control conditions.

The existence of a cytoskeletal network forming an undercoating to the axolemma and having attachments to the axolemma has been described morphologically (4,8,12,13). It is likely that some of the integral membrane proteins (e.g., the TTX binding protein) may be anchored or have interactions with the proteins of the cytoskeletal network. When TTX binds to its receptor sites, it may cause a conformational change in the binding site which could be "transmitted" throughout the binding protein. This conformational change could affect the interactions between the intrinsic membrane protein and the cytoskeletal

protein, thereby causing the cytoskeletal proteins to be released. An alternative explanation is as follows. We have previously suggested (2) that the protein release induced by the extracellular application of KCl may be due to the increase in the intracellular levels of calcium resulting from the KCl-induced depolarization. It is possible that TTX also increases the intracellular calcium levels thus causing protein release by a similar mechanism as the KCl-induced release. While these explanations of the TTX effect are speculative, the observations described in this paper suggest that tetrodotoxin and saxitoxin binding affect the state of the subaxolemmal network.

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