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The binding of tritiated tetrodotoxin to squid giant axons

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The binding of tetrodotoxin to squid giant axons was determined as a function of toxin concentration, using a tritiated toxin preparation of known radiochemical purity and specific activity. From the amount of saturable binding observed, the number of toxin binding sites thought to be sodium channels was found to be $553 \pm 119/\mu m^2$ of axon surface.

Introduction

During the recent years tritium labelled tetrodotoxin has been used to determine the number of tetrodotoxin binding sites in nerves from various animals. The number of specific binding sites is equal to the number of sodium channels if one assumes that the binding of one tetrodotoxin molecule completely blocks one channel (Hille 1970; Cuervo & Adelman 1970). Colquhoun, Henderson & Ritchie (1972) found the following binding site densities: $27/\mu m^2$ for rabbit vagus nerve, $16/\mu m^2$ for lobster walking leg nerve, $2.5/\mu m^2$ for garfish olfactory nerve. Similar figures were obtained by other authors; 22 and $28/\mu m^2$ for lobster walking leg nerve (Hafemann 1972; Barnola, Villegas & Camejo 1973), $3.9/\mu m^2$ for garfish olfactory nerve (Benzer & Raftery 1972). For squid giant axons the number of tetrodotoxin binding sites has not yet been directly determined. From an analysis of the time course of the tetrodotoxin action on squid axons the comparatively high figures of 200 and $500/m^2$ have been deduced (Keynes, Rojas & Taylor 1973; Keynes, Bezanilla, Rojas & Taylor 1974). In the present study, the concentration dependent uptake of tritiated tetrodotoxin of known purity and specific activity (Levinson 1974) was used to determine the density of toxin binding sites in squid giant axons.

Methods

The experiments were performed with giant axons 500–900 μm in diameter dissected from the hindmost stellar nerve in mantles from *Loligo forbesi*. The mantles had been stored for a few hours in ice-cold seawater before dissection. The axons were carefully cleaned of small nerve fibres over their whole length. Injured parts of the axon were tied off and excised. Diameters of the cleaned axon segments were measured every 2 cm and the surface area was calculated from the average diameter and the length of the axon segment. Binding studies were done with pooled axon segments. The total axon area used in a given sample depended on the concentration of toxin in the medium, smaller concentrations needing larger axon surfaces to give detectible radioactive uptakes. Areas were in the range 1.5–11 cm^2.

Fresh stock solutions of the labelled toxin (described in the preceding paper) in seawater were prepared daily. ^{14}C-labelled mannitol was added to these solutions as an extracellular

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space marker. The pooled axons were incubated in 1 ml of the toxin–mannitol mixture (diluted to the appropriate tetrodotoxin concentration) after draining away as much of the surrounding seawater as possible. The axons were incubated with the toxin for 10 min with intermittent agitation of the mixture. At the end of incubation, the axons were taken out of the fluid and briefly blotted on filter paper to remove as much extracellular fluid as possible.

After cutting off the ligatures on the axon ends, the axons were placed in a scintillation vial containing 1 ml sea water and the vial sealed and heated to 70 °C in a water bath for 4 min. This treatment has been shown to denature the specific tetrodotoxin binding in other tissues. For instance, the specific binding of electric organ particulate fraction (see Levinson, this volume, p. 337) was totally eliminated by heating the preparation for one 1 min at 60 °C. After denaturation, the axons were removed from the vial and discarded. Equilibrium of the extracellular space of the squid axons with the vial fluid during heating was demonstrated by comparing the space marker counts released from axons after 4 min of heating with the counts obtained from axons which were heated for 4 min and then incubated in the fluid overnight. No significant differences in the extracellular spaces of the axons were found in this comparison.

Ten ml scintillation fluid (Aquasol, New England Nuclear or Unisolve 1, Koch-Light Laboratories Ltd) was added to the vial and the sample counted by double labelling techniques in a Packard Tri-Carb Liquid Scintillation Spectrometer. Net tritium binding of the sample was determined as follows: the ratio of the count in the tritium scintillation channel to the count in the ^{14}C channel was determined in a sample of the stock solution. The ^{14}C space count in the axon sample was multiplied by this ratio to get the tritium count in the sample due to extracellular space, and these were subtracted from the total tritium count in the sample to give the actual tritium bound by the axons. Since the uptake of radioactivity at low toxin concentrations was small, background was minimized by the use of low potassium glass vials and the samples all counted for at least 100 min.

Counting the incubation fluid before and after soaking the axons showed that neither uptake of the toxin by the axons nor dilution of the incubating medium by the extracellular fluid changed the initial toxin concentration in the medium significantly. Extracellular spaces averaged 3.1 $\mu l/cm^2$ (s.e. $\pm$ 0.2 $\mu l/cm^2$), suggesting a 31 μm thick external layer which probably corresponds to the layer of Schwann cells and connective tissue (see Caldwell & Keynes 1960); the value of 31 μm is higher than the average value of 16.4 μm given by Shanes & Berman (1955) for axons from *Loligo pealii*.

All binding experiments were done in fresh or artificial seawater at ambient temperature. The artificial seawater contained 460 nM-NaCl, 10 mM-KCl, 11 mM-$CaCl_2$, 55 mM-$MgCl_2$ and 2.5 mM-$NaHCO_3$.

Results

The uptake of tritium label per square centimetre of axon surface as a function of concentration of tetrodotoxin is shown in figure 1. The data are somewhat scattered, but this variance is comparable to that observed in other low binding preparations (Colquhoun *et al.* 1972). This scatter most probably reflects a combination of statistical radioactivity counting error and biological variation. The presence of a linear component of uptake at high concentrations and the fact that the affinity constant of the toxin for the sodium channel has been shown to be about 3 nM (Cuervo & Adelman 1970) leads to the conclusion that a considerable non-

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specific component of uptake is present in the binding. Linear regression analysis of the points at high concentrations (represented by triangles) was used to determine this component and its intercept, which gives the amount of specific binding at saturation. Unfortunately, the scatter in the data was too great to allow use of the sensitive Scatchard plot to evaluate the saturable binding independently (see Levinson, this volume, p. 337). It should be mentioned that the regression line calculated probably gives a slightly low estimate for the intercept since some points were used in the calculation at low enough concentrations to still be affected by the rise of the saturable component. With this reservation, calculation of the number of binding sites based on a saturable binding of 23.7 ± 5.1 disintegrations min^{-1} cm^{-2} (figure 1) and a specific activity of the tetrodotoxin of 117.4 Ci/mol (preceding paper) gives a density of 553 ± 119 sites/μm^2.

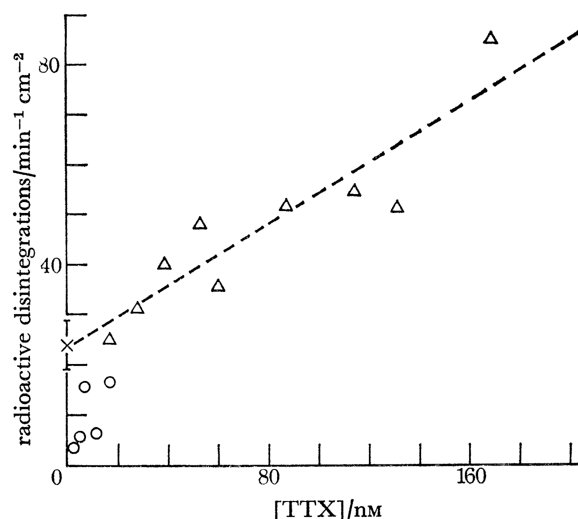

Figure 1. Binding of tritiated tetrodotoxin to squid giant axons. Radioactivity bound per square centimetre of axon surface is plotted against the concentration of tritiated tetrodotoxin in the incubation medium. The triangle points were used to calculate the regression line for linear binding at high tetrodotoxin concentrations. The computed regression line is shown, together with its intercept (23.7 d.min^{-1} cm^{-2}) and standard error of the intercept ($\pm$ 5.1 d.min^{-1} cm^{-2}).

Discussion

The estimated site density of 553/μm^2 is substantially larger than the site densities found in other nerves (see Introduction). The relatively large value is consistent in order of magnitude with the figures deduced from the kinetics of the tetrodotoxin action in squid axons. It is also consistent with an estimate of the number of sodium channels in the squid axon membrane (483/μm^2) based upon the maximum charge movement during the asymmetrical displacement currents (Keynes & Rojas 1974).

The squid giant axon represents an adaptation for high neural conduction velocity and it might be argued that both large fibre diameter and high channel density are necessary for such adaptation.

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References (Levinson & Meves)

Barnola, F. V., Villegas, R. & Camejo, G. 1973 Tetrodotoxin receptors in plasma membranes isolated from lobster nerve fibres. *Biochim. biophys. Acta* **298**, 84–94.

Benzer, T. I. & Raftery, M. A. 1972 Partial characterization of a tetrodotoxin-binding component from nerve membrane. *Proc. natn. Acad. Sci. U.S.A.* **69**, 3634–3637.

Caldwell, P. C. & Keynes, R. D. 1960 The permeability of the squid giant axon to radioactive potassium and chloride ions. *J. Physiol., Lond.* **154**, 177–189.

Colquhoun, D., Henderson, R. & Ritchie, J. M. 1972 The binding of labelled tetrodotoxin to non-myelinated nerve fibres. *J. Physiol., Lond.* **227**, 95–126.

Cuervo, L. A. & Adelman, W. J. 1970 Equilibrium and kinetic properties of the interaction between tetrodotoxin and the excitable membrane of the squid giant axon. *J. gen. Physiol.* **55**, 309–335.

Hafemann, D. R. 1972 Binding of radioactive tetrodotoxin to nerve membrane preparations. *Biochim. biophys. Acta* **266**, 548–556.

Hille, B. 1970 Ionic channels in nerve membranes. *Prog. Biophys. molec. Biol.* **21**, 1–32.

Keynes, R. D., Bezanilla, F., Rojas, E. & Taylor, E. R. 1974 The rate of action of tetrodotoxin in the squid giant axon. *Phil. Trans. R. Soc. Lond.* B **270**, 365–375 (this volume).

Keynes, R. D. & Rojas, E. 1974 Kinetics and steady state properties of the charged system controlling sodium conductance in the squid giant axon. *J. Physiol., Lond.* (in the Press.)

Keynes, R. D., Rojas, E. & Taylor, R. E. 1973 Saxitoxin, tetrodotoxin barriers, and binding sites in squid giant axons. *J. gen. Physiol.*, **61**, 267.

Levinson, S. R. 1974 The purity of tritiated tetrodotoxin as determined by bioassay. *Phil. Trans. R. Soc. Lond.* B **270**, 337–348 (this volume).

Shanes, A. M. & Berman, M. D. 1955 Kinetic of ion movement in the squid giant axon. *J. gen. Physiol.* **39**, 279–300.

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