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THE BINDING OF TETRODOTOXIN TO AXONAL MEMBRANE FRACTION ISOLATED FROM GARFISH OLFACTORY NERVE

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SUMMARY

The plasma membrane fraction which was isolated recently from garfish (*Lepisosteus osseus*) olfactory nerve and identified tentatively as axonal membrane has been studied for its interaction with ^{3}H-labelled tetrodotoxin. Tetrodotoxin binds to the membrane fraction with a dissociation constant of $5.5 \cdot 10^{-9}$ M and a maximal binding of 3.7 pmoles of tetrodotoxin per mg of membrane protein. The number of tetrodotoxin binding sites, calculated from the binding data, membrane composition, density and thickness, is 6.2 per μm^2 of surface area. These values are higher, but comparable because of further purification, than the values reported previously on garfish olfactory nerve total membrane.

INTRODUCTION

Garfish (*Lepisosteus osseus*) olfactory nerve is an excellent source of axonal membrane, relatively free from other cellular membranes and has already been the subject of several reports on its biochemical [1–5] and physiological [6, 7] properties. We have recently described the isolation and characterization of a plasma membrane fraction (Fraction I) from this nerve [8] and have tentatively identified this fraction as the axonal membrane based on its yield, $(Na^+–K^+)$-activated ATPase activity and by comparison of its biochemical properties to those of similar membrane fractions of lobster walking leg [9] and squid nerves [10–11]. In order to identify the membrane preparation unequivocally and to ascertain its functional integrity, its interaction with ^{3}H-labelled tetrodotoxin, which specifically blocks I_{Na+}, has been studied and the results are presented here. These results are compared with those obtained on a similar membrane fraction from lobster walking leg nerve whose interaction with tetrodotoxin has been reported [9].

EXPERIMENTAL

Isolation of axonal membrane fractions

The procedures used for the isolation of axonal membrane fractions from garfish

[8] and lobster nerves [9] have been reported previously. In the case of garfish nerve, it consisted of homogenization of the nerve in 0.25 M sucrose, 5 mM Tris buffer (pH 7.4), followed by centrifugation of the homogenate twice over 1.195 M sucrose in a SW 25.1 rotor for 90 min at 25 000 rev./min and finally separation of the total plasma membrane fraction, obtained as a sharp white band at the interface, by centrifugation over a discontinuous sucrose gradient system. Fraction I accumulated at the interface between 20 and 30 % sucrose and Fraction II at the 30 and 35 % sucrose. In the case of lobster nerve, the total plasma membrane fraction obtained on centrifugation of nerve homogenate in 0.33 M sucrose over 1.195 M sucrose was separated into two membrane fractions by centrifugation over a linear gradient between 0.66 and 1.195 M sucrose. The membrane fractions from lobster nerve were used fresh, and those from garfish after isolation were stored frozen up to 3 months in 0.250 M sucrose, 5 mM Tris buffer (pH 7.4). For tetrodotoxin binding studies the membrane suspensions were thawed quickly and the membrane material recovered after diluting 3 times with 10 mM Tris buffer (pH 7.4), by centrifugation in a Beckman rotor 30 at 29 000 rev./min for 30 min. The membranes were then washed once with the respective physiological solutions [9, 12], containing 10 mM Tris buffer at pH 7.4 and suspended in them at suitable membrane concentrations for binding studies.

Measurement of tetrodotoxin binding to the axonal membrane fractions

[^{3}H]Tetrodotoxin, labelled by the method of Wilzbach [13] and purified according to the procedure of Hafemann [14] as modified by Benzer and Raftery [4], was used. The concentration of tetrodotoxin was estimated by a mouse survival time assay [14]. The specific activity of the toxin was determined by measuring the radioactivity of the biologically active component as previously described [9] and was 13.0 Ci/mole. It had a radiochemical purity of 85 %. The experimental method eliminates the contribution of radioactive impurities to the [^{3}H]tetrodotoxin binding measurements. Thus in order to assay tetrodotoxin binding under a given set of conditions, duplicate samples of membrane were used, one of each pair serving to measure non-specific binding. Binding of [^{3}H]tetrodotoxin was calculated by subtracting the radioactivity (cpm/mg protein) present in pellets of membranes pre-incubated with an excess (50 times that of the [^{3}H]tetrodotoxin) from that found in pellets of membranes incubated with [^{3}H]tetrodotoxin only. Incubation with tetrodotoxin and centrifugation afterwards of the membrane suspension were carried out at 4 °C in polycarbonate centrifuge tubes (IEC No. 2055). The concentration of [^{3}H]tetrodotoxin used for determination of the maximal binding to the membrane was 100 nM.

RESULTS AND DISCUSSION

The binding of [^{3}H]tetrodotoxin (pmoles/mg membrane protein) to the Membrane Fraction I, which corresponds to axonal membrane, was plotted as a function of the concentration of free tetrodotoxin in the incubation medium and is shown in Fig. 1. From the double reciprocal plot of the data, shown in the insert, a dissociation constant of $5.5 \cdot 10^{-9}$ M for the binding of tetrodotoxin to the membrane was obtained. This is in good agreement with those obtained for a similar axonal membrane fraction from lobster nerve [9] ($4.5 \cdot 10^{-9}$ M) and for total membrane fraction from garfish olfactory nerve, $6.0 \cdot 10^{-9}$ M by Henderson and Wang [3] and $8.3 \cdot 10^{-9}$ M

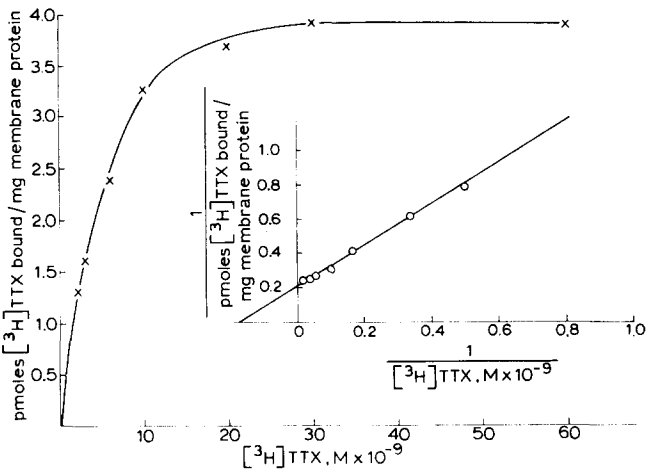

Fig. 1. Binding curve of [^{3}H]tetrodotoxin to axonal membrane isolated from garfish olfactory nerve. pmoles of [^{3}H]tetrodotoxin bound per mg membrane protein is plotted against the concentration of free [^{3}H]tetrodotoxin. The insert shows the reciprocal plot of the binding data.

by Benzer and Raftery [4]. Electrophysiological experiments provide values for the binding of tetrodotoxin to axonal membranes of live nerves: $3.3 \cdot 10^{-9}$ M for squid giant axon [15], $3.5 \cdot 10^{-9}$ M for the non-myelinated fibers of rabbit vagus nerve [16] and $10.1 \cdot 10^{-9}$ M for whole garfish olfactory nerve [7]. A maximal binding of 3.7 pmoles of tetrodotoxin per mg membrane protein was obtained for the axonal membrane fraction when the uptake of tetrodotoxin was measured from a tetrodotoxin concentration of 100 nM. From this value and from the data (Table I) on the density of the membrane fraction (1.094 g/ml), the percentage composition of protein

TABLE I

COMPARISON OF THE PROPERTIES OF PLASMA MEMBRANE FRACTIONS ISOLATED FROM GARFISH OLFACTORY AND LOBSTER LEG NERVES

Tetrodotoxin binding was measured from respective physiological solutions containing 100 nM [^{3}H]tetrodotoxin.

	Fraction I		Fraction II	
	Garfish	Lobster	Garfish	Lobster
Density	1.094	1.072	1.142	1.124
Protein (%)	34	24	55	39
Total lipid (%)	66	76	45	61
(Na^+–K^+)-ATPase*	4.6	44	1.4	13.9
Tetrodotoxin binding**	3.7±0.3 (8)	9.5±0.6 (24)	1.6	3.4
Dissociation constant (K_D) (nM)	5.5	4.5		

* μmoles Pi/mg protein per h.

** pmoles/mg membrane protein. Values given are mean ±SD; number of measurements is shown in parentheses.

(34 %), and from the thickness of the membrane (75 Å) obtained from the high resolution electron micrographs, it can be calculated that there are 6.2 binding sites per μm^2 of surface area. This value is higher, but comparable because of further purification, than the value (3.9 binding sites per μm^2) reported by Benzer and Raftery [4] on garfish olfactory nerve total membrane.

The Fraction II membrane, which is chemically and enzymatically different from Fraction I, was also found to bind tetrodotoxin, although less than half on a mg protein basis (Table I). A similar membrane fraction obtained from lobster nerve was also found to bind tetrodotoxin in smaller concentration as compared to the axonal membrane fraction. Recent electrophysiological measurements on squid nerve fibers [17] have indeed shown the presence of tetrodotoxin sensitive sodium channels in the Schwann cell plasma membrane, the significance of which is under investigation.

The present results on the binding of tetrodotoxin to membrane fraction isolated from garfish olfactory nerve confirms that Fraction I corresponds to the axonal membrane and that it retains its functional integrity, at least with respect to its tetrodotoxin binding and receptor sites, in addition to its structural integrity as indicated by electron microscopic [8] and X-ray diffraction studies [18]. This membrane fraction is obtained in high yield (20 mg/g of wet nerve) and should be useful for chemical and biochemical studies of excitable membranes. A similar fraction from lobster nerve has recently been obtained and its interaction with tetrodotoxin has been reported. A comparison of some of the properties (Table I) of axonal membrane preparations from these two sources indicates a low protein to lipid ratio in both cases as compared with other types of plasma membrane. It also shows that the number of tetrodotoxin binding sites per μm^2 and the (Na^+-K^+)-ATPase activity are different for the two membrane preparations. A direct relationship appears to exist between the number of tetrodotoxin binding sites and (Na^+-K^+)-ATPase activities. Further comparative studies on other properties, such as the polypeptide pattern and the lipid composition of these two axonal membrane preparations, are being carried out.

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