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[^{3}H]-tetracaine binding on rat synaptosomes and sodium channels

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1 [^{3}H]-tetracaine binding was studied in a rat synaptosomal preparation. [^{3}H]-tetracaine bound to a single class of binding sites with a mean K_D of 188 ± 28 nM and a mean maximal binding capacity of 13 ± 0.7 pmol mg^{-1} protein.

2 [^{3}H]-tetracaine binding was inhibited by tetracaine, procaine and by β-adrenoceptor blocking agents which possess local anaesthetic properties.

3 [^{3}H]-tetracaine binding was not modified by neurotoxins interacting specifically with the sodium channels.

Introduction

In recent years, toxins, specific for the fast Na^+ channels, have proved very useful in analysing the structural and functional properties of these channels. These toxins belong to different classes and their actions differ (Bidard *et al.*, 1984, Postma & Catterall, 1984): tetrodotoxin and saxitoxin block the Na^+ channels, whereas batrachotoxin and veratridine activate them, while sea anemone and scorpion toxins slow down their inactivation. Some of these toxins have been labelled and their ability to bind to Na^+ channel receptors has been investigated; among them: [^{3}H]-ethylene diamine tetrodotoxin ([^{3}H]-en-TTX; Lombet *et al.*, 1981, 1982; Frelin *et al.*, 1983; Renaud *et al.*, 1983), [^{3}H]-saxitoxin ([^{3}H]-STX; Catterall & Coppersmith, 1981), [^{3}H]-batrachotoxinin A 20α-benzoate ([^{3}H]-BTX-B; Postma & Catterall, 1984; Creveling *et al.*, 1983) and [^{125}Iodine]-sea anemone toxin II ([^{125}I]-ATX II, Vincent *et al.*, 1980). The results obtained suggest that Na^+ channels have at least three receptor sites: tetrodotoxin and saxitoxin bind at one receptor site, batrachotoxin and veratridine at another, scorpion and sea anemone toxins at yet a third site. Except [^{3}H]-BTX-B, the radiolabelled toxins used for these studies were not, until now, commercially available.

Recently, [^{3}H]-tetracaine has been introduced for labelling sites involved in local anaesthetic effects. The interference by local anaesthetics with Na^+ channels, thus reducing sodium permeability, is well known (Ritchie & Greengard, 1966; Courtney, 1975). Moreover, the inhibition of [^{3}H]-BTX-B binding by local anaesthetics has been described by Postma & Catterall (1984) in rat synaptosomes and by Creveling *et al.* (1983) in a guinea-pig cerebral cortex preparation. On the other hand, Catterall (1981) and Lombet *et al.* (1981) found no effect on [^{3}H]-STX binding and [^{3}H]-en-TTX binding, respectively.

In this work, our aim was to describe [^{3}H]-tetracaine binding in a rat synaptosomal preparation and to study the inhibition of this binding by various drugs including neurotoxins, local anaesthetics and β-adrenoceptor blocking agents known for their local anaesthetic effects (Hellenbrecht *et al.*, 1973). Calcium antagonists such as verapamil, for which local anaesthetic side-effects have been described (Hay & Wadsworth, 1982) were also tested.

Methods

Preparation of synaptosomes

Synaptosomes were prepared from whole rat brain according to the method described by Tamkun & Catterall (1981). Briefly, the brains of four male Wistar rats were removed and homogenized in ice-cold 0.32 M sucrose, 5mM K_2HPO_4, pH 7.4 (10% wet weight/volume) with ten strokes of a motor-driven teflon glass homogenizer. The homogenate was centrifuged at 1,000 *g* for 10 min. The supernatant was kept and the pellet was resuspended in the homogenizing solution (10% v/v) and recentrifuged at 17,000 *g*

for 60 min. The resulting pellet was suspended in 9 ml of 0.32 M sucrose, 5 mM K_2HPO_4, pH 7.4, and then layered on top of three discontinuous gradients consisting of 7 ml layers of 1.2, 1.0, 0.8, 0.6, 0.4 M sucrose in 5 mM K_2HPO_4, pH 7.4. The gradients were centrifuged at 100,000 *g* (SW28 rotor, Beckman centrifuge) for 105 min. The synaptosomes were collected at the 1.0–1.2 M sucrose interface and diluted to a 0.32 M sucrose concentration by the dropwise addition of 5 mM K_2HPO_4, pH 7.4, with constant stirring. The suspension was centrifuged at 40,000 *g* for 45 min. The final pellet was resuspended in incubation medium (composition, mM: KCl 7.4, $MgSO_4$ 0.8, glucose 5.5, Hepes-Tris (pH 7.4) 50 and choline chloride 130) to a final concentration of about 10 mg protein ml^{-1}. The whole procedure was performed at 4°C. The synaptosomes were divided into 250 µl aliquots and slowly frozen on dry ice before storage in liquid nitrogen.

Protein concentration was determined by the method of Lowry *et al.* (1951).

Binding assay

Binding assay was performed by incubating 20 µg of synaptosomal protein with increasing concentrations of [^{3}H]-tetracaine (50 to 500 nM; NEN 38 Ci $mmol^{-1}$) in incubation medium, pH 7.4 (total volume = 150 µl). Incubation was carried out at 25°C for 20 min. Bound and free ligands were separated by rapid filtration through Whatman GF/B filters. The filters were rapidly washed with 20 ml of ice-cold buffer solution (composition, mM: KCl 5.4, $MgSO_4$ 0.8, glucose 5.5, Hepes-Tris (pH 7.4) 50 and choline chloride 130) and transferred to counting vials containing 10 ml scintillation mixture (Packard TM 299). Radioactivity was measured in a Packard counter at 43% efficiency.

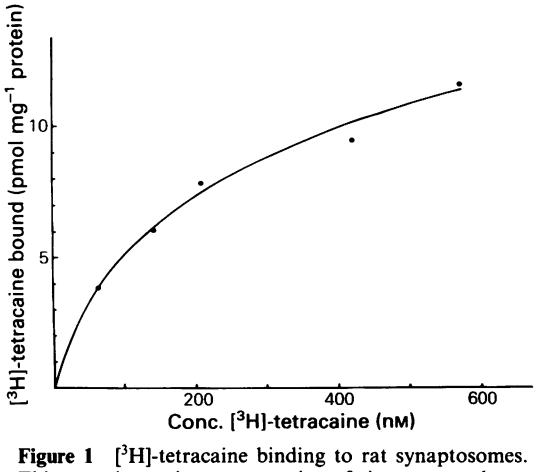

Figure 1 [^{3}H]-tetracaine binding to rat synaptosomes. This experiment is representative of six separate determinations.

Binding in the presence of tetracaine 10^{-4} M was considered as non-specific binding. Specific binding amounted to 70% total binding.

In competition experiments, various concentrations of the competing drugs were incubated under the same conditions with 20 µg synaptosomal protein and 80 nM [^{3}H]-tetracaine.

Half-maximal inhibition of specific binding gave IC_{50} values from which inhibition constants (K_i) were calculated:

$$K_i = \frac{IC_{50}}{1 + \frac{(tetra)}{K_D}}$$

where (tetra) is the concentration of [^{3}H]-tetracaine and K_D the dissociation constant of this ligand.

Results

[^{3}H]-tetracaine binding to rat synaptosomes

Under experimental conditions with ligand concentrations ranging from 50 to 500 nM, [^{3}H]-tetracaine binding was saturable (Figure 1). Scatchard analysis (Figure 2) of the data gave a straight line, showing that this ligand binds to a single class of receptors. Six experiments were performed, four with four different synaptosomal preparations and two with the same one. Experimental data (means ± s.e.mean) gave a K_D value of 188 ± 28 nM and a maximal binding capacity

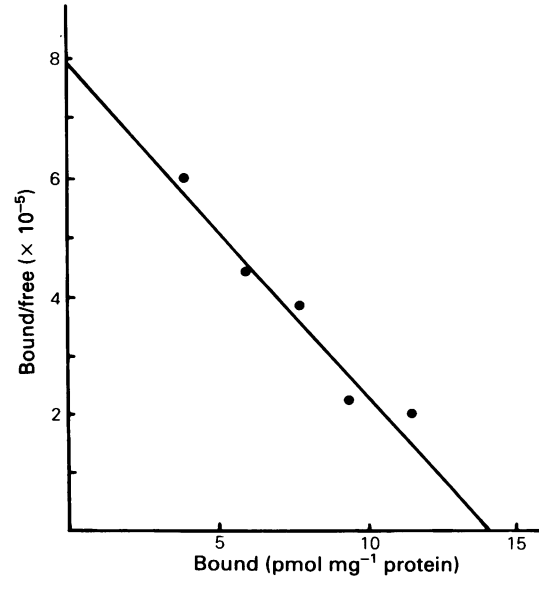

Figure 2 Scatchard analysis of the specific binding of [^{3}H]-tetracaine shown in Figure 1.

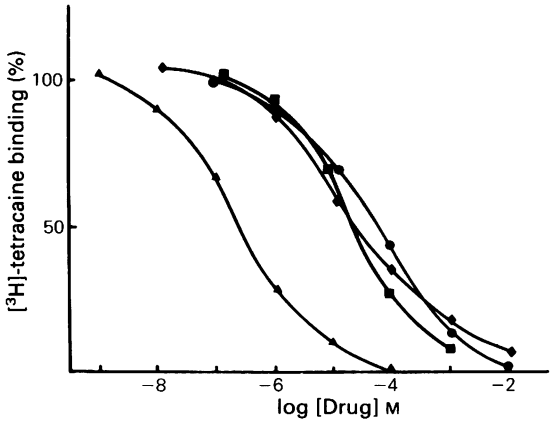

Figure 3 Inhibition of [^{3}H]-tetracaine binding to rat synaptosomes by tetracaine (▲), procaine (◆), propranolol (●) and alprenolol (■). Data are means of three determinations, each point being done in triplicate.

of 13 ± 0.7 pmol mg^{-1} protein. Figure 1 shows a typical saturation curve representative of six separate determinations and Figure 2 the Scatchard analysis of the same curve.

Inhibition of [^{3}H]-tetracaine binding by competitive drugs

The inhibition by various drugs of [^{3}H]-tetracaine binding to a rat synaptosomal preparation is shown in Figure 3 and Table 1. For each drug three determinations were performed with each point being done in triplicate. Tetracaine and procaine inhibited [^{3}H]-tetracaine binding with IC_{50} values of 0.3 and 40 μM, respectively. Propranolol and alprenolol, two β-adrenoceptor blocking agents which also have local anaesthetic properties, had similar effects on [^{3}H]-tetracaine binding (IC_{50} values of 70 and 26 μM, respectively). Practolol, a β-adrenoceptor blocking agent without any anaesthetic activity, had no effect on [^{3}H]-tetracaine binding. Tetrodotoxin, batrachotoxin and anthopleurin A, neurotoxins which act specifically on sodium channels, were found not to modify [^{3}H]-tetracaine binding. We noted that protoveratrines A and B, alkaloids which induce persistent activation of Na^+ channels, did not inhibit but rather increased [^{3}H]-tetracaine binding; with these drugs, at concentrations 10^{-5} M to 10^{-3} M, we observed a 150 to 200% increase in binding compared with the control (Figure 4). The same effect was observed for clonidine with a 150% increase in [^{3}H]-tetracaine binding, but only at a concentration of 10^{-3} M. The two calcium channel inhibitors tested, diltiazem and verapamil, had no effect on [^{3}H]-tetracaine binding.

Table 1 Inhibition of [^{3}H]-tetracaine binding to rat synaptosomes

Drug	IC_{50} (μM)	K_i (μM)
Tetracaine	0.3 ± 0.03	0.21 ± 0.02
Procaine	40 ± 3	28 ± 2
Propranolol	70 ± 8	49 ± 5
Alprenolol	26 ± 4	18 ± 3
Practolol	no effect	
Tetrodotoxin	no effect	
Batrachotoxin	no effect	
Protoveratrine A	↑ binding	
Protoveratrine B	↑ binding	
Clonidine	↑ binding	
Diltiazem	no effect	
Verapamil	no effect	

Data are means ± s.e.mean of three determinations.
↑ = increase.

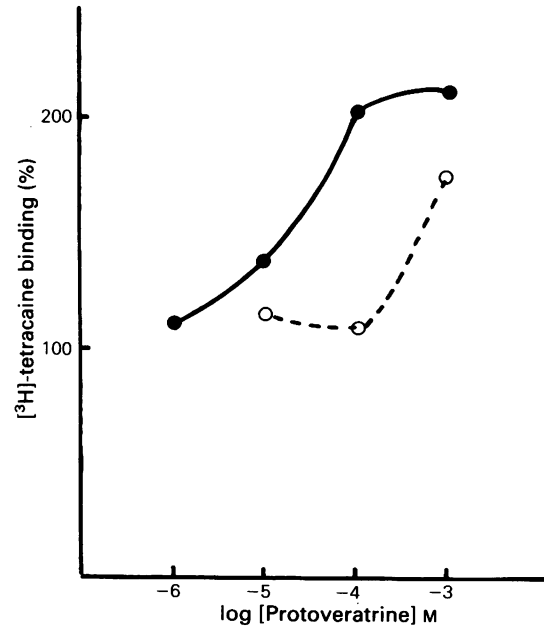

Figure 4 Increase in [^{3}H]-tetracaine binding to rat synaptosomes induced by protoveratrine A (●) and protoveratrine B (○). Data are means of three determinations, each point being done in triplicate.

Discussion

In our experiments with rat synaptosomes, [^{3}H]-tetracaine was found to bind with high affinity to a single class of binding sites. Hence, this ligand allows the study of relatively low concentrations of receptors (in the picomolar range).

The inhibition of [^{3}H]-tetracaine binding by unlabelled tetracaine and procaine gives an order of potency similar to that of local anaesthetic properties, tetracaine being more potent than procaine. Only the β-adrenoceptor blocking agents which possess local anaesthetic properties inhibited [^{3}H]-tetracaine binding the order of potency being similar to that described by Hellenbrecht *et al.* (1973) for their ability to depress nerve action potential in the frog isolated sciatic nerve: alprenolol was more active than propranolol and practolol had no effect.

Clonidine acts on [^{3}H]-tetracaine binding by increasing the amount of bound radiolabelled ligand. Starke *et al.* (1972) have shown that clonidine is as potent a local anaesthetic as procaine; a membrane-stabilizing type of mechanism might be envisaged for the hypotensive effect. However, in our experiments, clonidine did not behave like other local anaesthetics.

Tetrodotoxin and batrachotoxin, which have opposite effects on Na^+ channels had no effect on [^{3}H]-tetracaine binding. Catterall *et al.* (1981) and Ritchie & Rogart (1977) have described at least three separate receptor sites for sodium channels. Tetrodotoxin binds to one site and inhibits ion flux through the sodium channel (Ritchie & Rogart, 1977); batrachotoxin and veratridine act at another site to cause persistent activation of the channel (Catterall, 1980); other toxins, such as scorpion or sea anemone toxin, bind to a third site and slow down sodium channel inactivation (Catterall, 1977; 1980; Catterall *et al.*, 1981).

Postma & Catterall (1984) and Creveling *et al.* (1983) proposed that batrachotoxin and local anaesthetics act at the same site. Indeed, [^{3}H]-BTX-B binding is inhibited by local anaesthetics. However, our results are not in agreement with this hypothesis. On the other hand, our results with tetrodotoxin accord with those of Henderson *et al.* (1973) and Lombet *et al.* (1981) who observed no inhibition of saxitoxin and tetrodotoxin binding, respectively, by local anaesthetics.

Interference between tetracaine and protoveratrines requires an explanation. Many years ago, it was shown that the interaction of local anaesthetics with the Na^+ channel depended upon the frequency and duration of Na^+ channel activation and inactivation (Khodorov *et al.*, 1976; Hille, 1977); this suggests that the binding of a local anaesthetic increases in proportion to the number of Na^+ channels present in the open form. Persistent activation of Na^+ channels induced by protoveratrine might explain the increase in [^{3}H]-tetracaine binding.

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