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Anti-anginal arylalkylamines and sodium channels: [^{3}H]-batrachotoxinin-A 20-α-benzoate and [^{3}H]-tetracaine binding

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1 [^{3}H]-batrachotoxinin-A 20-α-benzoate ([^{3}H]-BTX-B) and [^{3}H]-tetracaine are useful ligands for the study of sodium channels.

2 Inhibition of their binding by various anti-anginal drugs was tested on a rat synaptosomal preparation and on a heart membrane preparation.

3 Diphenylalkylamines and structurally related drugs inhibited [^{3}H]-BTX-B binding in both the synaptosomal preparation and heart membrane preparation. They were almost inactive on [^{3}H]-tetracaine binding.

4 These results suggest that activity of arylalkylamines could be mediated by an interaction on the sodium channel.

Introduction

Calcium antagonists differ widely both on the experimental level and in clinical practice. According to Spedding (1985), there are three different classes of calcium antagonists. Class III comprises the diphenylalkylamines and also drugs with saturated rings such as perhexiline. Perhexiline is a potent anti-anginal drug but its mechanism of action is not yet clear. Similarly, cinnarizine and flunarizine are potent vasodilator drugs, but their calcium antagonist activity is poor. On the other hand, McNeal *et al.* (1985) showed that cinnarizine, flunarizine and also prenylamine inhibited the binding of [^{3}H]-batrachotoxinin-A 20-α-benzoate ([^{3}H]-BTX-B), a ligand specific for a site associated with the gating mechanism of the sodium channels. Flunarizine, for example, was some 50 times more potent than dihydropyridines.

In this work, we have explored the possible effect on the sodium channel, of phenylalkylamines with anti-anginal activities; [^{3}H]-BTX-B and [^{3}H]-tetracaine were used to test this hypothesis and binding assays were performed on a rat synaptosomal preparation and on a heart membrane preparation. We also included other vasodilator drugs such as amiodarone, perhexiline and a series of IPS drugs (Institut de Pharmacologie Strasbourg, Leclerc *et al.*, 1982) structurally related to perhexiline. Formulae are given in Figure 1.

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Methods

Preparation of synaptosomes

Synaptosomes were prepared from whole rat brain according to the method described by Postma & Catterall (1984). Briefly, the brains of four male Wistar rats were removed and homogenized in ice-cold 0.32 M sucrose, 5 mM K_2HPO_4, pH 7.4 (10% wet weight/volume) with ten strokes of a motor-driven teflon glass homogenizer. The whole procedure was performed at 4°C. The homogenate was centrifuged at 1,000 *g* for 10 min and the supernatant was retained; the pellet was resuspended in the homogenizing solution (10% volume/volume) and recentrifuged as above. The two supernatants were combined and centrifuged 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 5mM 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 0.32 M sucrose 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 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 50 mM HEPES-

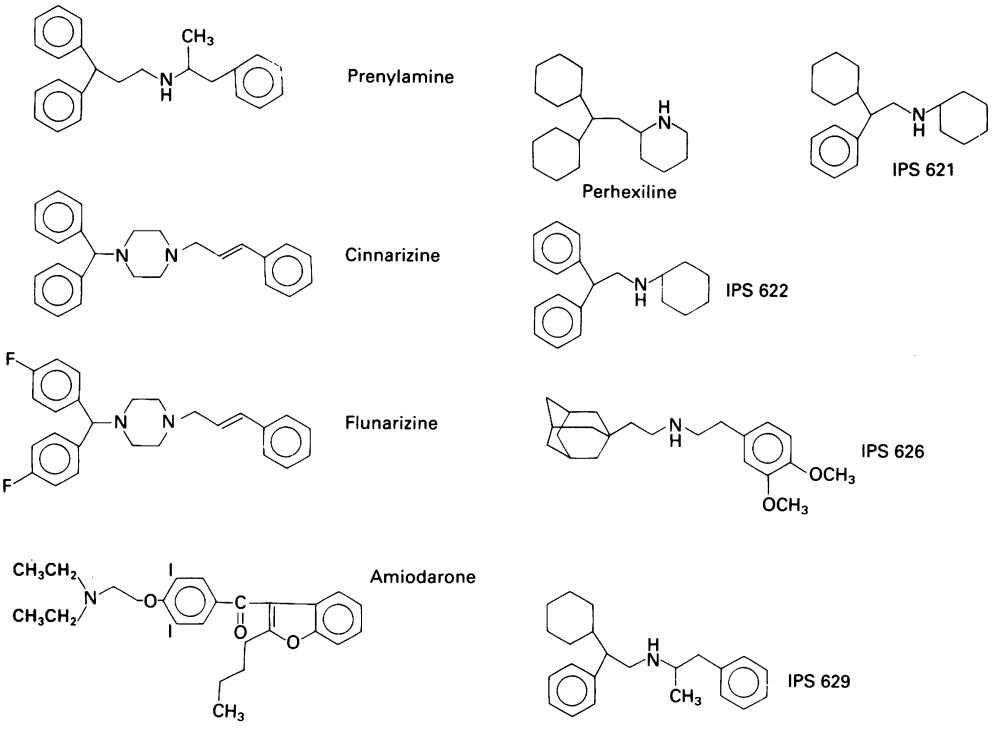

Figure 1 Structure of IPS drugs used.

Tris (pH 7.4) and 130 mM choline chloride (incubation medium) to a final protein concentration of about 10 mg protein ml^{-1}. 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).

Heart membrane preparation

Rat hearts were homogenized in 7 volumes of ice-cold 0.32 M sucrose, 5 mM K_2HPO_4 with a polytron tissue disrupter (setting 6, for 20s). The homogenate was centrifuged at 10,000 *g* for 10 min and the pellet was washed with the same buffer. After a second centrifugation, the pellet was suspended in 1.72 M sucrose, 5 mM K_2HPO_4, pH 7.4 (20 ml g^{-1} wet weight). This suspension was tranferred into cellulose nitrate tubes (24 ml/tube) and covered with a 15 ml layer of 0.2 M sucrose, 5 mM K_2HPO_4. The gradients were centrifuged at 100,000 *g* (SW 28 rotor Beckman centrifuge) for 70 min. The interface fraction was collected and diluted to 0.32 M sucrose by the dropwise addition of 5 mM K_2HPO_4, pH 7.4. The suspension was centrifuged at 95,000 *g* for 60 min. The final pellet was resuspended in incubation medium (5.4 mM KCL, 0.8 mM $MgSO_4$, 5.5 mM glucose, 50 mM HEPES-Tris, pH 7.4 and 130 mM choline chloride) to a final concentration of about 6 mg ml^{-1} and frozen until the binding assays.

[^{3}H]-tetracaine binding

Binding assay was performed by incubating 20 µg of synaptosomal protein or 200 µg protein for heart membrane preparation 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 (5.4 mM KCl, 0.8 mM $MgSO_4$ 5.5 mM glucose, 50 mM HEPES-Tris pH 7.4 and 130 mM choline chloride) and transferred to counting vials containing 10 ml scintillation mixture (Packard TM 299). Radioactivity was measured in a Packard counter at 43% efficiency. Binding in the presence of tetracaine 100 µM was considered as non specific. Specific binding was 70% of total binding.

In competition experiments, various concentrations of the competing drugs were incubated under the same conditions with 20 µg synaptosomal protein or 200 µg protein for heart preparation and 80 nM [^{3}H]-tetracaine. For each drug, three tests were performed, each point being determined in triplicate.

[^{3}H]-batrachotoxinin-A 20-α-benzoate binding

[^{3}H]-BTX-B (50 Ci mmol^{-1}) was obtained from New England Nuclear. Binding assay was performed according to the method described by Postma & Catterall (1984). For both synaptosomal and heart membrane preparation, 200 μg of protein were incubated with increasing concentrations of [^{3}H]-BTX-B (10 to 150 nM) in incubation medium (5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 50 mM HEPES adjusted to pH 7.4 with Tris, 130 mM choline chloride). Incubation was conducted in the presence of tetrodotoxin (1 μM), scorpion toxin (Leiurus scorpion toxin, Sigma ref. V 5251; 100 μg ml^{-1}) and bovine serum albumin (BSA 1 mg ml^{-1}). Tetrodotoxin (TTX) blocked sodium channels and had no effect on the binding of [^{3}H]-BTX-B. It was included in order to prevent ion movements through sodium channels activated by scorpion toxin (Catterall *et al.*, 1981). Unlike the membrane preparations from neonatal rat cardiac ventricles, preparations from adult rats revealed a high-affinity TTX-binding site (Renaud *et al.*, 1983). Consequently, the same concentration of TTX was added both to the rat heart membrane preparation and to synaptosomes. Total volume was 150 μl. After incubation at 36°C for 30 min, bound and free ligands were separated by rapid filtration through Whatman GF/C filters. The filters were rapidly washed with 20 ml washing medium (163 mM choline chloride, 5 mM HEPES-Tris pH 7.4, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, BSA 1 mg ml^{-1}) and transferred to counting vials containing 10 ml scintillation mixture (Packard TM 299). Radioactivity was measured in a Packard counter at 43% efficiency. Non-specific binding was determined in the presence of 1 mM protoveratrine A. Specific binding was 75% of total binding. In competition experiments, various concentrations of the drugs were incubated under the same conditions with 200 μg protein and 15 nM [^{3}H]-BTX-B. For each drug three tests were performed, each point being determined in triplicate.

Analysis of data

The equilibrium dissociation constants (K_D) and the maximal number of binding sites (B_{max}) were calculated for [^{3}H]-BTX-B and [^{3}H]-tetracaine from Scatchard plots. The experimental data given in the paper are means ± s.e. mean. In competition experiments on the inhibition of [^{3}H]-BTX-B and [^{3}H]-

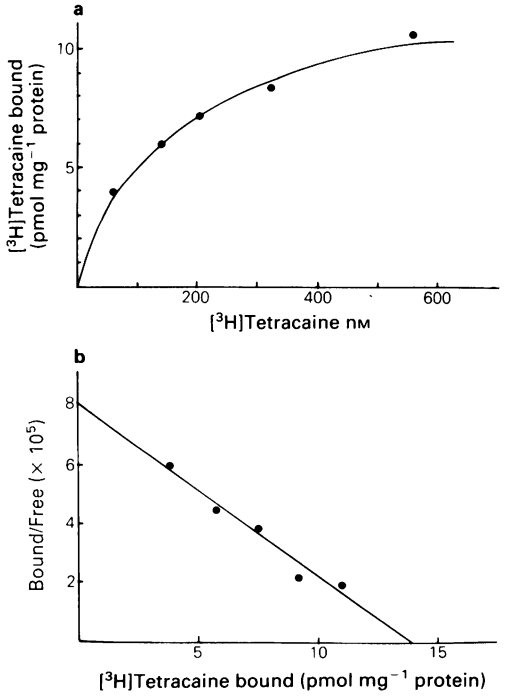

Figure 2 Specific [^{3}H]-tetracaine binding on rat synaptosomes. (a) A typical saturation curve representative of six separate assays; (b) Scatchard analysis of the same curve.

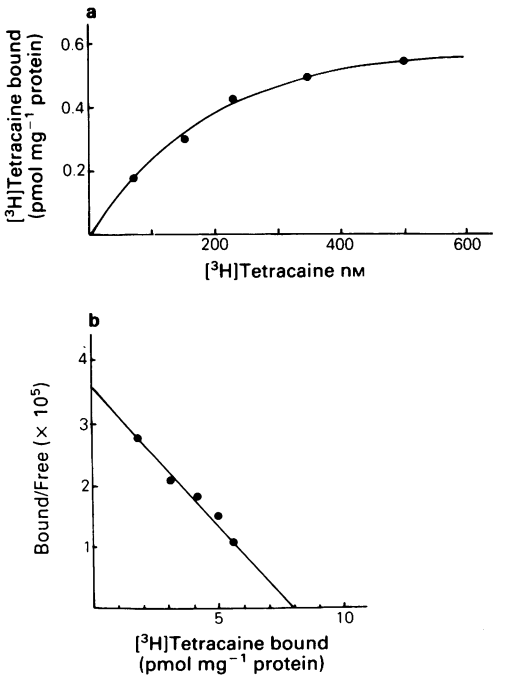

Figure 3 Specific [^{3}H]-tetracaine binding on the rat heart membrane preparation. (a) A typical saturation curve representative of six separate assays; (b) Scatchard analysis of the same curve.

tetracaine by various drugs, half maximal inhibition of specific binding (IC_{50}) was determined. Data given in the paper are means of three determinations.

Results

[^{3}H]-tetracaine binding

Specific binding to rat synaptosomes and heart membrance preparation: Under experimental conditions, with ligand concentrations ranging from 50 to500 mM, [^{3}H]-tetracaine binding was saturable both for synaptosomes and heart (Figures 2 and 3). Scatchard analysis of the data gave a straight line, showing that this ligand binds to a single class of receptors. Six experiments were performed with different synaptosomal preparations and six more with six different heart membrane preparations. Experimental data (means ± s.e.means) gave a mean K_D of 188 ± 28 nM for synaptosomes and a mean maximum binding capacity of 13 ± 0.7 pmol mg^{-1} protein. For the heart preparation, mean K_D was 262 ± 36 nM and mean maximum binding capacity, 8 ± 0.7 pmol mg^{-1} protein. Figure 2a shows a typical saturation curve representative of the six determinations on synaptosomes, and Figure 2b the Scatchard analysis of the same curve. Similarly, Figure 3b is the Scatchard analysis of Figure 3a, a typical saturation curve representative of the six determinations performed with the heart membrane preparation.

Inhibition of [^{3}H]-tetracaine binding by diphenylalkylamines and structurally related drugs is shown in Table 1. All the drugs tested, excepted tetracaine and procaine, were very weak inhibitors, with IC_{50} ranging from 0.03 mM to more than 1 mM. Similar inhibition potencies were observed whether binding was performed on synaptosomes or on the heart membrane preparation.

[^{3}H]-batrachotoxinin-A 20 α-benzoate binding

Specific binding to rat synaptosomes and heart membrane preparation: Under experimental conditions, with ligand concentrations ranging from 10 to 150 nM, [^{3}H]-BTX-B binding was saturable (Figures 4 and 5). Scatchard analysis of the data gave a straight line, indicating a single class of binding sites for BTX-B, with a mean K_D of 83 ± 13 nM and a mean maximum binding capacity (B_{max}) of 1.8 ± 0.2 pmol mg^{-1} protein for synaptosomes, whole for the heart membrane preparation, the corresponding values were 44 ± 8 nM and 0.5 ± 0.06 pmol mg^{-1} protein. Figures 4a and 5a show typical saturation curves of six separate experiments performed respectively on synaptosomes and on heart membrane preparation, while Figures 4b and 5b are the Scatchard analysis of the same curves.

Inhibition of [^{3}H]-BTX-B binding by diphenylalkylamines and structurally related drugs is shown in Table 1. All the drugs tested inhibited [^{3}H]-BTX-B binding. The most potent diphenylalkylamines were flunarizine, prenylamine and cinnarizine, with IC_{50}s of respectively 27, 32 and 79 nM. The coronary vasodilator drugs, amiodarone and perhexiline, were also potent inhibitors of [^{3}H]-BTX-B binding with IC_{50}s of 75 nM and 290 nM respectively. In the IPS series, IPS 621, IPS 626 and IPS 629 were equally potent (IC_{50} 180, 240 and 110 nM respectively). IPS 622, which only differs from IPS 621 by an aromatic ring instead of a saturated one, was about 8 times less potent with an IC_{50} of 1500 nM.

Comparison of [^{3}H]-BTX-B binding on synap-

Table 1 Inhibition of [^{3}H]-batrachotoxinin-A 20α-benzoate ([^{3}H]-BTX-B) and [^{3}H]-tetracaine binding to rat synaptosomal and heart membrane preparations.

	Inhibition of [^{3}H]-BTX-B binding IC_{50} (M)		*Inhibition of [^{3}H]-tetracaine binding* IC_{50} (M)	
Drug	*Synaptosomes*	*Heart*	*Synaptosomes*	*Heart*
Batrachotoxin	1.6×10^{-7}	4.8×10^{-7}	$> 10^{-4}$	
Tetracaine	1.8×10^{-7}	2.2×10^{-6}	3×10^{-7}	3.1×10^{-7}
Procaine	3.3×10^{-6}	2.9×10^{-6}	4×10^{-5}	4.4×10^{-6}
Lidocaine	4.3×10^{-5}	7.8×10^{-5}	3.5×10^{-4}	2.0×10^{-4}
Prenylamine	3.2×10^{-8}	2.4×10^{-7}	2.0×10^{-4}	8.0×10^{-4}
Cinnarizine	7.9×10^{-8}	1.3×10^{-6}	$> 10^{-4}$	8.0×10^{-5}
Flunarizine	2.7×10^{-8}	3.2×10^{-7}	$> 10^{-4}$	3.5×10^{-5}
Amiodarone	7.5×10^{-8}	4.8×10^{-6}	$> 10^{-4}$	$> 10^{-3}$
Perhexiline	2.9×10^{-7}	1.0×10^{-5}	$> 10^{-4}$	2.2×10^{-4}
IPS 621	1.8×10^{-7}	3.7×10^{-6}	2.2×10^{-4}	$> 2.10^{-4}$
IPS 622	1.5×10^{-6}	2.3×10^{-6}	2.5×10^{-3}	$> 10^{-3}$
IPS 626	2.4×10^{-7}	3.2×10^{-6}	$> 10^{-4}$	$> 10^{-4}$
IPS 629	1.1×10^{-7}	2.6×10^{-7}	$> 10^{-4}$	$> 10^{-4}$

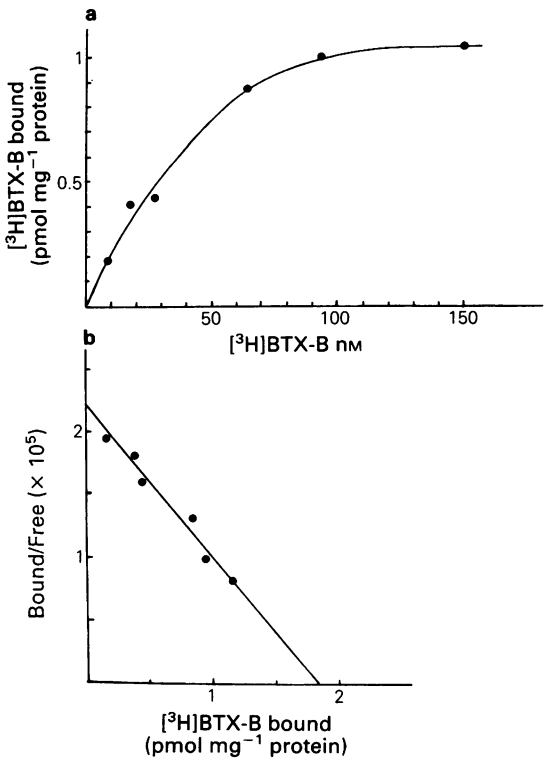

Figure 4 Specific [^{3}H]-batrachotoxinin-A 20 α-benzoate. ([^{3}H]-BTX-B) binding on rat synaptosomes. (a) A typical saturation curve representative of six separate assays; (b) Scatchard analysis of the same curve.

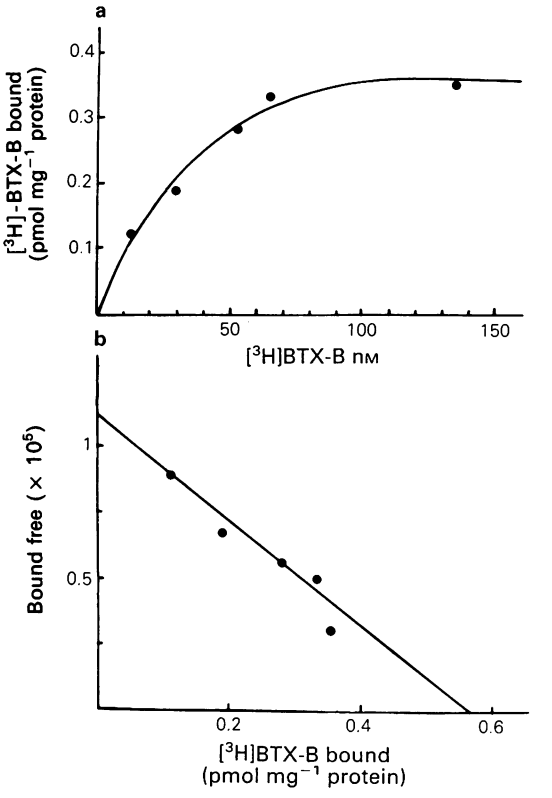

Figure 5 Specific [^{3}H]-batrachotoxinin-A 20 α-benzoate ([^{3}H]-BTX-B) binding on the rat heart membrane preparation. (a) A typical saturation curve representative of six separate assays; (b) Scatchard analysis of the same curve.

tosomes and on the heart membrane preparation showed that the drugs were generally more active on synaptosomes but the order of potencies was the same. We found good correlation between the two bindings ($r = 0.749$, $P < 0.01$).

Discussion

In this work, we tested the ability of diphenylalkylamines and structurally related drugs to inhibit [^{3}H]-tetracaine and [^{3}H]-BTX-B binding. Binding assays were performed on a rat synaptosomal preparation and on a membrane heart preparation. The synaptosomal preparation, made up of vesicular elements, had retained functional channels, as shown by Tamkun & Catterall (1980). This was probably not the case for the heart membrane preparation for which the gating system might have been impaired. However, we found for [^{3}H]-BTX-B binding, good correlation between results with synaptosomes and the heart membrane preparation. So, the potencies of drugs with cardiac and vascular activities can be evaluated on a synaptosomal preparation as well as on a heart membrane preparation. However, it is possible that differences may exist between sodium channels in cardiac muscle and those in neuronal membranes; moreover, binding sites associated with vasodilatation might also differ.

Batrachotoxin has been shown to interact specifically with the sodium channel (Catterall, 1980), so [^{3}H]-BTX-B was used as ligand for the study of drugs interacting with sodium channels, especially local anaesthetics (Creveling *et al.*, 1983, Postma & Catterall, 1984; McNeal *et al.*, 1985). With [^{3}H]-tetracaine, we investigated interference with the binding of this ligand, by various drugs acting on the sodium channel (Grima *et al.*, 1985). These various publications gave conflicting results: [^{3}H]-tetracaine binding was not modified by batrachotox in (Grima *et al.*, 1985); conversely, local anaesthetics were able to compete with [^{3}H]-BTX-B binding (Creveling *et al.*,

1983; Postma & Catterall, 1984).

The present work showed no evident inhibition of [^{3}H]-tetracaine binding by the various drugs tested (except for tetracaine and procaine) but an obvious inhibitory potency as regards [^{3}H]-BTX-B binding. As previously assumed, [^{3}H]-tetracaine and [^{3}H]-BTX-B appear not to bind at the same site.

Our results on [^{3}H]-BTX-B binding inhibition by prenylamine, cinnarizine and flunarizine are in agreement with those obtained by McNeal *et al.* (1985). These drugs are more potent inhibitors of [^{3}H]-BTX-B binding than tetracaine, procaine and lidocaine. Flunarizine and lidocaine were found to possess similar anaesthetic activities when tested on the rat phrenic nerve (Hay *et al.*, 1982); however, flunarizine is 1600 times more potent than lidocaine in inhibiting [^{3}H]-BTX–B binding. There is therefore no correlation between the inhibition of [^{3}H]-BTX-B binding and local anaesthetic activities.

It is obvious from the present results that the arylalkylamines, in addition to their calcium inhibitor activities, interact with the sodium channel. This was described for prenylamine by Sada (1978) who tested, on a papillary muscle preparation, the ability of this drug to reduce the maximal rate of rise of action potential (V_{max}) considered to be a measure of sodium inward current (Weidmann, 1955). As regards the mechanism of amiodarone activity, little is known. Mason *et al.* (1983) observed its effect on V_{max}, tested on a papillary muscle preparation. They showed that amiodarone blocks cardiac sodium channels and, selectively, channels in the inactivated state, only slightly affecting open and resting channels. This tallies well with our results on [^{3}H]-BTX-B binding inhibition by amiodarone.

Perhexiline, another anti-anginal drug, and IPS compounds, structurally related to perhexiline, also inhibit [^{3}H]-BTX-B binding; their activity could be mediated by an interaction with the sodium channels.

It seems that these drugs could act through a well-defined receptor site; but it must be recalled that diphenylalkylamines are lipophilic and basic drugs and could bind unspecifically to all membranal systems (Rodenkirchen, 1982; Spedding, 1985).

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