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THE MEMBRANE STABILIZING ACTIVITY OF β-ADRENOCEPTOR LIGANDS*

QUANTITATIVE EVALUATION OF THE INTERACTION OF PHENOXYPROPANOLAMINES WITH THE [^{3}H]BATRACHOTOXININ A 20-α-BENZOATE BINDING SITE ON VOLTAGE-SENSITIVE SODIUM CHANNELS IN RAT BRAIN

ADRIAAN P. IJZERMAN, ASHA NAGESSER and ANJA GARRITSEN
Division of Medicinal Chemistry, Center for Bio-Pharmaceutical Sciences, PO Box 9502, 2300 RA Leiden, The Netherlands

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Abstract—The interaction of 12 phenoxypropanolamines, all ligands with high affinity towards β-adrenoceptors, with the [^{3}H]batrachotoxinin A 20-α-benzoate ([^{3}H]BTX-B) binding site on voltage-sensitive sodium channels in a rat brain synaptosomal preparation, was studied as a measure for local anaesthetic activity. All derivatives are capable of displacing [^{3}H]BTX-B from its specific binding site, penbutolol displaying the highest affinity. Multiple regression analyses were performed, correlating biological activity (pK_i values) with physicochemical parameters. Lipophilicity is of prime importance in the established regression equations, but steric factors are relevant as well. Comparisons were made with analogous equations relating β_1- and β_2-adrenoceptor affinity with physicochemical parameters, leading to the conclusion that "cardioselective" β-adrenoceptor ligands appear to have fewer membrane stabilizing properties.

There is ample evidence that, besides β-adrenoceptor occupation, lipophilic β-adrenoceptor ligands exert a, usually undesired, effect on the cell membrane, the so-called membrane stabilizing activity (MSA) or local anaesthetic activity. As several cell types may be involved, many expressions of these effects, unrelated to β-adrenoceptor occupation, have been described in various *in vivo* and *in vitro* pharmacological assays.

Thus, Hellenbrecht *et al.* [1] demonstrated that local anaesthetic activity (isolated frog nerve) and myocardial conduction velocity (frog heart) were markedly influenced by nine β-adrenoceptor ligands, which effects were linearly correlated with the lipophilicities of the derivatives. Similar conclusions, to mention only a few recent findings, were drawn with respect to the immobilization of human sperm [2], the corneal penetration in the rabbit eye [3], the inhibition of [^{3}H]noradrenaline uptake in rat brain synaptosomes [4], and the effects on maximum upstroke velocity of the action potential in guinea-pig papillary muscles [5]. Furthermore, in radioligand binding studies, the influence on non-specific binding of radiolabeled β-adrenoceptor antagonists, such as [^{3}H]dihydroalprenolol, is dependent on the lipophilicity of the displacing agent [6], at least in tissue preparations with high non-specific binding.

Characteristically, the potencies of the β-adrenoceptor ligands with regard to these effects usually are more than three decades lower than their respective affinities towards the β-adrenoceptor. Moreover, no stereospecificity is observed, the *l*- and *d*-stereoisomers of propranolol, for instance, being equiactive.

The molecular pharmacological basis for this membrane stabilizing activity has been and still is a matter of debate. It has been suggested that β-adrenoceptor ligands interact with membrane phospholipids, such as phosphatidylcholine [7, 8]. On the other hand, as local anaesthetic activity is often explained in terms of blockade of the voltage-dependent sodium channels (in neurons), it is conceivable that β-adrenoceptor ligands are capable of interacting with this channel, resulting in "membrane stabilization".

Recently, it was established that the binding of a radiolabeled neurotoxin derivative, [^{3}H]batrachotoxinin A 20-α-benzoate ([^{3}H]BTX-B), to (a site of) the voltage-sensitive sodium channel in rat or guinea-pig brain preparations is inhibited by compounds with local anaesthetic activities [9–11], including some β-adrenoceptor agents.

Previously, we quantitatively documented the interaction of β-adrenoceptor ligands with both β_1- and β_2-adrenoceptors by radioligand binding studies [12, 13]. In the present paper we evaluate, again in a quantitative fashion, the binding of 12 phenoxypropanolamines to the voltage-dependent sodium channel, as measured by their displacement of the specific [^{3}H]BTX-B binding to a rat brain synaptosomal preparation. Physicochemical properties of the compounds are taken into account and are related to the biological effects, in order to derive clues that provide a molecular basis for the membrane stabilizing activity of β-adrenoceptor ligands.

* In memoriam to Dr T. Bultsma.

MATERIALS AND METHODS

Preparation of synaptosomes. Synaptosomes were prepared from rat brain by a modification of the method of Gray and Whittaker [14], as described by Postma and Catterall [9]. Briefly, the brains of male Wistar rats (200–250 g, CPB, TNO, Zeist, The Netherlands) were removed, homogenized in ice-cold 0.32 M sucrose (pH 7.4) and centrifuged at 1000 *g* for 10 min. The supernatant was centrifuged at 17,000 *g* for 45 min. The resuspended pellet (0.32 M sucrose/5 mM K_2HPO_4/KH_2PO_4 pH 7.4) was layered onto a discontinuous sucrose gradient, and centrifuged at 100,000 *g* for 105 min. Synaptosomes were collected from the 1.0–1.2 M sucrose interface, resuspended (final sucrose concentration 0.32 M), and centrifuged at 40,000 *g* for 45 min. The final pellet was resuspended in ice-cold buffer consisting of 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 130 mM choline chloride, and 50 mM HEPES–Tris (pH 7.4). Aliquots were frozen on dry ice, and subsequently stored in liquid nitrogen. Binding characteristics remained constant for more than three months.

[^{3}H]BTX-B binding assay. All assays were performed in duplicate in a final volume of 250 µl containing the following components in a HEPES buffer (50 mM HEPES, adjusted to pH 7.4 with Tris base):
(a) 100 µl of various concentrations of β-adrenoceptor ligands, or 100 µl veratridine (final concentration 500 µM to obtain nonspecific binding), or 100 µl buffer;
(b) 50 µl [^{3}H]BTX-B (2–3 nM);
(c) 50 µl of a crude scorpion toxin *Leiurus quinquestriatus* solution (final concentration 200 µg/ml);
(d) 50 µl of a synaptosomal suspension in HEPES buffer (2 mg/ml).

Incubations were carried out for 30 min at 37°, and were terminated by the addition of 2 ml ice-cold HEPES buffer to the incubation tubes. The synaptosomes were immediately collected on glass-fiber filters (Whatman GF/C) under vacuum and washed three times with 2 ml HEPES buffer. Radioactivity retained at the filters was counted, following drying of the filters at 70° for 45 min, in 4 ml OptiPhase MP counting liquid at 55% efficiency on a LKB 1214 RackBeta liquid scintillation spectrometer. Specific binding was defined as the difference between radioactivity bound in the absence and presence of 500 µM veratridine or 200 µM aconitine, which yielded identical results.

Protein determination. Protein was determined by the method of Lowry *et al.* using bovine serum albumin as a standard [15].

Data analysis. The method of computer assisted data analysis following the law of mass-action has previously been described [16].

K_I and K_D values are given with approximated SE.

QSAR parameters. Log *P* values (octanol/water) of the aromatic moiety of the ligands (i.e. the corresponding, substituted benzenes) were calculated, according to the hydrophobic fragmental system [17].

The steric branching parameter (Sb) for all substituents was calculated according to Austel *et al.* [18].

Multiple regression analysis. Computer assisted multiple regresssion analyses were performed, which yielded the regression equations, together with statistic parameters, adjusted for the degrees of freedom. The regression coefficients are given with their standard errors.

Materials. [^{3}H]BTX-B (45.2 Ci/mmol) was purchased from New England Nuclear (Dreieich, F.R.G.). Veratridine and scorpion venom *(Leiurus quinquestriatus)* were obtained from Sigma Chemical Company (St. Louis, MO).

(−)-Penbutolol (sulphate, Hoechst, Amsterdam, The Netherlands), (±)-propranolol, (±)-atenolol, and (±)-practolol (hydrochlorides and base, respectively, ICI, Macclesfield, Cheshire, U.K.), (±)-alprenolol, (±)-H87/07, and (±)-metoprolol (hydrochlorides and tartrate, respectively, AB Hässle, Mölndal, Sweden), and (±)-Kö 589, (±)-Kö 592 (toliprolol), (±)-Kö 1124, (±)-Kö 1350, and Kö 1411 (hydrochlorides and oxalate, respectively, Boehringer, Ingelheim, F.R.G.) were gifts. All other chemicals were of analytical grade.

RESULTS

In Fig. 1 the behaviour of three β-adrenoceptor ligands with respect to the displacement of specific [^{3}H]BTX-B binding is shown. Penbutolol displays the highest affinity (in the low micromolar range), whereas practolol is not very effective in displacing [^{3}H]BTX-B from its binding sites in our rat brain synaptosomal preparation. All three drugs do not influence non-specific [^{3}H]BTX-B binding. As documented in a previous paper [19], [^{3}H]BTX-B binding in this synaptosomal preparation is effectively displaced by batrachotoxin and veratridine (results not shown). K_I values are similar to those obtained by others [20], and the binding of [^{3}H]BTX-B is characterized by relatively high affinity and capacity (K_D ~ 90 nM, B_{max} ~ 5 pmol/mg protein). In Table 1 the displacement characteristics of the mentioned and nine other β-adrenoceptor blocking agents are represented by their K_I and pK_I values, respectively,

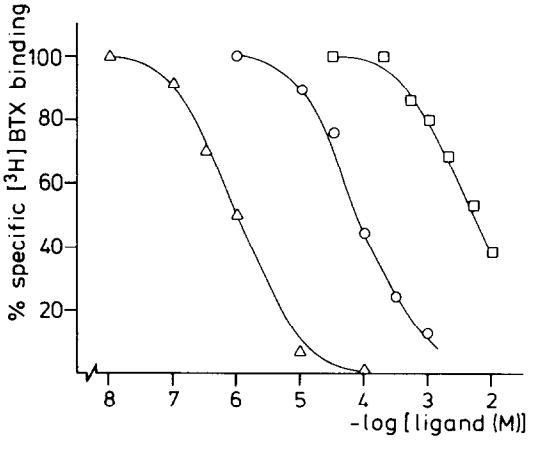
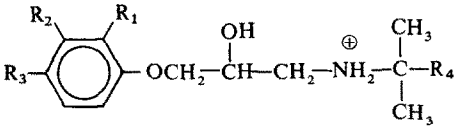


Fig. 1. Displacement of specific [^{3}H]BTX-B binding from rat brain synaptosomes by penbutolol (△), metoprolol (○), and practolol (□); a representative experiment is shown ([^{3}H]BTX-B = 2.1 nM).

Table 1. K_I values* of β-adrenoceptor ligands with respect to [^{3}H]BTX-B binding to synaptosomes of rat brain



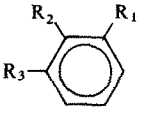
Name	R_1	R_2	R_3	R_4	K_I (μM)	pK_I (M)
1. Penbutolol	C_5H_9†	H	H	CH_3	0.95 ± 0.21	6.02
2. Kö 1124	H	$CH_3CHC_2H_5$	H	H	4.0 ± 0.6	5.40
3. Alprenolol	$CH_2CH{=}CH_2$	H	H	H	7.8 ± 0.6	5.11
4. Propranolol	—CH=CH—CH=CH	—	H	H	14 ± 4	4.85
5. Kö 1411	$OCH_2C{\equiv}CH$	H	H	H	20 ± 2	4.70
6. Kö 592	H	CH_3	H	H	35 ± 5	4.46
7. Kö 589	CH_3	H	H	H	49 ± 11	4.31
8. Metoprolol	H	H	$CH_3OC_2H_4$	H	81 ± 12	4.09
9. Kö 1350	CH_2OH	H	H	H	180 ± 30	3.74
10. H 87/07	H	H	$CH_3OC_2H_4O$	H	250 ± 20	3.60
11. Practolol	H	H	CH_3CONH	H	6,000 ± 700	2.22
12. Atenolol	H	H	H_2NCOCH_2	H	9,500 ± 1,500	2.02

* K_I value of each ligand is the result of at least three experiments performed in duplicate (pK_I = − log K_I).
† C_5H_9: cyclopentyl.

together with their structural formulas. From computer-assisted data analysis it was revealed that the interaction between the β-adrenoceptor ligands and the [^{3}H]BTX-B binding site is best described by the assumption of one single binding site. This is in agreement with the finding that all displacement curves have slope factors ("pseudo" Hill coefficients) which do not differ significantly from unity (results not shown).

From Table 1 it is obvious that all ligands are capable of displacing specific [^{3}H]BTX-B binding, but with potencies that vary considerably. In an attempt to explain this diversity in affinities, we have focused on the structural and physicochemical characteristics of the derivatives. All ligands are phenoxypropanolamines, having an identical aliphatic side chain (one exception: penbutolol has a tert.-butylamino function, the others are isopropylamino derivatives) The pK_a values of the phenoxypropanolamines range from 9.2 to 9.5 [21], the amounts of cations approximating 100% at pH 7.4. On the assumptions that the cations are the active ionic species interacting with the sodium channel, and that *l*- and *d*-stereoisomers are equipotent [10], no further corrections with respect to K_I values were made. Thus, in this case, total drug concentrations refer to active drug concentrations, and variation in biological effect should be ascribed almost exclusively to variation in substitution patterns of the aromatic nucleus within this class of β-adrenoceptor ligands. Therefore, we have calculated some physicochemical parameters of substituted benzenes, corresponding to the aromatic moieties of the phenoxypropanolamines. In Table 2 the calculated lipophilicities of the substituted benzenes, and the calculated steric parameters for the various substituents are delineated. Lipophilicities are the log *P* values (octanol/water), calculated according to the hydrophobic fragmental system [17]. In a previous report we have shown that experimentally

Table 2. Calculated physicochemical parameters of substituted benzenes, corresponding to the aromatic nucleus of the β-adrenoceptor ligands



R_1	R_2	R_3	log P_{calc}	Sb_{R_1}	Sb_{R_2}	Sb_{R_3}
C_5H_9	H	H	4.253	3.0	0.0	0.0
H	$CH_3CHC_2H_5$	H	4.098	0.0	4.0	0.0
CH_2CHCH_2	H	H	3.215	3.0	0.0	0.0
—CH=CH—CH=CH	—	H	3.295	1.0	1.0	0.0
$OCH_2C{\equiv}CH$	H	H	2.412	3.0	0.0	0.0
H	CH_3	H	2.541	0.0	1.0	0.0
CH_3	H	H	2.541	1.0	0.0	0.0
H	H	$CH_3OC_2H_4$	1.984	0.0	0.0	3.0
CH_2OH	H	H	1.178	2.0	0.0	0.0
H	H	$CH_3OC_2H_4O$	2.105	0.0	0.0	3.0
H	H	CH_3CONH	1.251	0.0	0.0	4.0
H	H	H_2NCOCH_2	0.384	0.0	0.0	4.0

determined log P values (as far as known) closely match the calculated log P values for these substituted benzenes [12]. Steric influences are quantified by the so-called steric branching parameter (Sb), which largely accounts for branching of a substituent rather than for "width" or "length" [18].

First, we have considered the correlation between the pK_I values of the compounds and their respective lipophilicities. For all twelve ligands the following equation was derived by (multiple) regression analysis

$$pK_I = 0.95(\pm 0.12) \log P_{calc} + 1.89\ (\pm 0.31)$$
$$N = 12 \quad r = 0.9332 \quad s = 0.4488 \qquad (1)$$
$$F = 67.403$$

Equation (1) is graphically represented in Fig. 2.

The omission of penbutolol, being the only *N*-tert.butyl substituted derivative, did not significantly influence the equation

$$pK_I = 0.94(\pm 0.14) \log P_{calc} + 1.91(\pm 0.35)$$
$$N = 11 \quad r = 0.9128 \quad s = 0.4719 \qquad (2)$$
$$F = 44.938$$

Although the contribution of lipophilicity to binding explains more than 80–85% of the observed variance (r^2), a further improvement of the equations could be achieved by the introduction of Sb_{R_3}, the steric branching parameter for the substituents *para* to the aliphatic side chain. The result is shown for all twelve ligands

$$pK_I = 0.71(\pm 0.12) \log P_{calc} - 0.24(\pm 0.08)Sb_{R_3} + 2.77(\pm 0.37) \qquad (3)$$
$$N = 12 \quad r = 0.9647 \quad s = 0.3465$$
$$F = 66.898$$

Although the inclusion of Sb_{R_1} (*ortho*) instead of Sb_{R_3} also yields an improved equation, the combined use of both steric parameters in regression analysis clearly demonstrates that only the contribution of Sb_{R_3} is significant (results not shown).

Neither the inclusion of Sb_{R_2} (*meta*) and $(\log P)^2$, nor the use of other parameters such as Hammett's σ(electronicity) improve the quality of the regression equations.

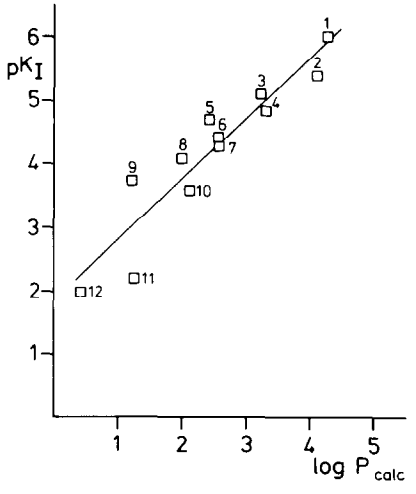


Fig. 2. Linear relationship between calculated lipophilicities and experimentally determined pK_I values for [^{3}H]BTX-B displacement of 12 β-adrenoceptor ligands, according to equation (1) in the text. Numbering of compounds is according to Table 1.

DISCUSSION

The inhibition of [^{3}H]BTX-B binding by local anaesthetics has been documented as an indirect allosteric mechanism [9]. Although, as in the present study (see Fig. 1 and Table 1), displacement studies in equilibrium suggest a merely competitive interaction between [^{3}H]BTX-B and compounds with local anaesthetic properties, the rate of [^{3}H]BTX-B dissociation was shown to be increased in the presence of these derivatives, altogether indicative for an indirect allosteric mechanism. Thus, there appears to be a local anaesthetic (LA) receptor distinct from the [^{3}H]BTX-B binding site. In the discussion we will further refer to this putative complex of proteins as the [^{3}H]BTX-B/LA binding site, as present on the voltage-sensitive sodium channel. From the established regression equations the major conclusions to be drawn are:

(1) lipophilicity is the predominant factor that determines the affinity of the phenoxypropanolamines for the [^{3}H]BTX-B/LA binding site on the voltage-sensitive sodium channel;
(2) the substituent *para* to the aliphatic side chain, exerts two effects on the affinity: it influences lipophilicity (as do all substituents), and, second, by steric hindrance, it negatively influences sodium channel affinity

As mentioned in the Introduction, lipophilicity has been found to be of prime importance in all studies dealing with membrane stabilization by β-adrenoceptor ligands. To our knowledge this is the first report to interpret and evaluate this phenomenon quantitatively by radioligand binding studies on the sodium channel. In Table 3 we have ranked the relative potencies of a number of β-adrenoceptor antagonists in various functional pharmacological tests, and compared these with the relative affinities for the [^{3}H]BTX-B/LA binding site. Although it can be concluded from the respective correlation coefficients that a fair parallellism between sodium channel binding and functional parameters exists, two remarks have to be made. First, in functional studies it is likely that the transport of the active substance from the organ bath into the tissue, a phenomenon which is governed by lipophilicity as well, is an important factor. Second, a good correlation *per se* does not necessarily mean that all non specific functional effects of β-blockers are indeed mediated through an interaction with the voltage-sensitive sodium channel.

In agreement with our second conclusion from the regression equations, Ban and coworkers, analysing the effects of β-adrenoceptor blocking agents on the maximum upstroke velocity on the action potential in guinea-pig papillary muscles, concluded that *para* substituted derivatives leave the sodium channels in

Table 3. Relative potencies of β-adrenoceptor ligands in sodium channel binding (column 1) and functional pharmacological tests (columns 2–5)

	K_{rel}*	Loc. an.* (frog)	Myocard.* cond. (frog)	NA-upt.* (rat)	Neg. inotr.* (guinea-pig)
Propranolol	1.00	1.00	1.00	1.00	1.00
Alprenolol	1.79	1.44	1.27	0.45	0.89
Kö 592	0.40	0.14	0.37	-	0.28
Metoprolol	0.17	—	—	0.07	—
Practolol	0.002	0.002	0.004	0.11	—
Atenolol	0.001	—	—	0.006	0.006
		r† = 0.98	r† = 0.97	r† = 0.65	r† = 0.87

* K_{rel}: $K_{I,propanolol}/K_{I,\beta\text{-blocker}}$, according to Table 1.

Loc. an.: local anesthesia of sciatic frog nerve, 10 min incubation, according to ref. 1.

Myocard. cond.: inhibition of myocardial conduction velocity in frog heart strips, 10 min incubation, according to ref. 1.

NA-upt.: inhibition of [^{3}H]noradrenaline uptake in rat brain synaptosomes, according to ref. 4.

Neg. inotr.: negative inotropic action on isolated left atria of guinea-pig heart, according to ref. 29.

† r: correlation coefficient with respect to the data in column 1 (K_{rel}).

this organ preparation more quickly than observed for compounds unsubstituted in this direction [5]. Furthermore, these authors did not establish marked differences between the *N*-tert. butyl and *N*-isopropyl substituted phenoxypropanolamines, which places reliance on the inclusion of penbutolol (*N*-tert. butyl substituted) in the regression equations.

How do these results compare with β-adrenoceptor affinity? In a previous study [13] we have presented regression equations for the β_1- and β_2-adrenoceptor affinities of a similar class of 14 phenoxypropanolamines, derived in an analogous way. For reasons of comparison, the best equations for β_1-, β_2-adrenoceptor, and sodium channel affinity are represented together in Table 4 (without the standard errors of the regression coefficients and the statistic parameters).

Obviously, the lipophilic environments of the binding sites, whether on the β-adrenoceptor or on the sodium channel, do not differ markedly. For the β-adrenoceptor it has been suggested that tryptophan moieties on the macromolecule are responsible for the lipophilic interaction [22], which idea could be of relevance for the definition of the [^{3}H]BTX-B/LA binding site on the sodium channel.

The steric aspects of the respective binding sites are clearly different. All three substituent positions determine β-adrenoceptor affinity, whereas the sodium channel is only sensitive to substitution *para* to the aliphatic side chain. Thus, the introduction of a bulky *para* substituent induces β_1-selectivity (by decreasing β_2-adrenoceptor affinity more drastically), together with a less prominent local anaesthetic activity. Therapeutically, this particular combination of effects is usually strongly favoured [23].

Table 4. Physicochemical parameters and their regression coefficients determining β_1- and β_2-adrenoceptor, and sodium channel affinity of phenoxypropanolamines

	log P_{calc}	Sb_{R_1}	Sb_{R_2}	Sb_{R_3}	Intercept
β_1	0.81	0.11	−0.35	−0.17	5.96
β_2	0.77	0.30	−0.25	−0.51	6.03
Sod. ch.	0.71	—	—	−0.24	2.77

A third point of discussion are the values of the intercepts in the three regression equations: *ca.* 6.0 for both β-adrenoceptors, in contrast with a value of 2.8 for the sodium channel. Obviously, the oxypropanolamine side chain is far better accommodated for by the β-adrenoceptor binding sites than by the active site on the sodium channel. [^{3}H]BTX-B, due to the presence of a tertiary amino group in the molecule, is positively charged (monovalent cation) at pH 7.4, as are the oxypropanolamines. Thus, the charged amino function in the β-adrenoceptor ligands *per se* seems mandatory for affinity at the [^{3}H]BTX-B/LA binding site. Among other explanations, it can therefore be reasoned that the distance between the lipophilic moiety and the charged amino function is not fully adequate for optimal affinity towards the sodium channel.

The above speculations are strongly stressed by the findings of McNeal *et al.* [10] with respect to the "classical" local anaesthetics. Benzocaine, lacking an amino function protonated at physiological pH, has virtually no effect in the [^{3}H]BTX-B binding assay, whereas the lipophilic local anaesthetics with secondary or tertiary amino functions, like hexylcaine and dibucaine, respectively, display affinities comparable to penbutolol in the present study. Local anaesthetics bearing a quaternary ammonium group (the methiodides) tend to be less active than the corresponding tertiary analogues, although no regular pattern is observed. Hence, it can be speculated that the charged amino function should preferably be a protonated one. Interestingly, besides the β-adrenoceptor antagonists and the local anaesthetics, a great variety of other classes of drugs inhibit [^{3}H]BTX-B binding, including calcium antagonists, tricyclic antidepressants and tranquillizers, such as phenothiazines and butyrophenones. All active substances share a lipophilic region and a (charged) amino function.

From these considerations it would be interesting to further explore the nature of the lipophilic region and the distance between this region and the charged amino function. A starting point could be pimozide (a D_2 dopamine antagonist) which was shown to be very effective in displacement studies with [^{3}H]BTX-B [11]. Its IC_{50} value of 50 nM is even lower than the K_d value of [^{3}H]BTX-B itself (~ 90 nM). The log P value of pimozide is 5.56, and from its pK_a value it can be derived that more than 90% of this compound is protonated at physiological pH.

A final point to discuss is the phenomenon of non-specific binding in radioligand binding studies performed with labeled lipophilic β-adrenoceptor ligands, like [^{3}H]dihydroalprenolol. The high extent of non-specific binding with this radioligand observed in membrane preparations of skeletal muscles [24, 25] could be overcome by the inclusion of phentolamine (10^{-4} M) in the incubation medium [26]. Since skeletal muscles are richly endowed with the voltage-sensitive sodium channel [27], and as phentolamine is a rather effective displacer of [^{3}H]BTX-B with an IC_{50} value of 8.4 μM, it can be imagined that non-specific binding of [^{3}H]dihydroalprenolol has to be interpreted, at least in this case, in terms of sodium channel binding. Of course, the high amount of non-specific binding that can be displaced non stereospecifically, observed with [^{3}H]dihydroalprenolol in cell types (whether membrane preparations or intact cells) without a significant population of voltage-sensitive sodium channels will demand another explanation [6, 28].

In conclusion, it is suggested that the molecular basis of the so-called membrane stabilizing or local anaesthetic properties of β-adrenoceptor ligands is found in their interaction with the voltage-sensitive sodium channel. This interaction is dependent on lipophilic and steric parameters of the ligands, which can be used to separate β-adrenoceptor affinity from membrane stabilizing activity.

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