[³H]-lifarizine, a high affinity probe for inactivated sodium channels

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1 [³H]-lifarizine bound saturably and reversibly to an apparently homogeneous class of high affinity sites in rat cerebrocortical membranes ($K_d = 10.7 \pm 2.9$ nM; $B_{max} = 5.10 \pm 1.43$ pmol mg⁻¹ protein).

2 The binding of [³H]-lifarizine was unaffected by sodium channel toxins binding to site 1 (tetrodotoxin), site 3 (α -scorpion venom) or site 5 (brevetoxin). Furthermore, lifarizine at concentrations up to 10 μ M had no effect on [³H]-saxitoxin (STX) binding to toxin site 1. Lifarizine displaced [³H]-batrachotoxinin-A 20- α -benzoate (BTX) binding with moderate affinity ($pIC_{50} = 7.31 \pm 0.24$) indicating an interaction with toxin site 2. However, lifarizine accelerated the dissociation of [³H]-BTX and decreased both the affinity and density of sites labelled by [³H]-BTX, suggesting an allosteric interaction with toxin site 2.

3 The binding of [³H]-lifarizine was voltage-sensitive, binding to membranes with higher affinity than to synaptosomes (pIC_{50} for cold lifarizine = 7.99 ± 0.09 in membranes and 6.68 ± 0.14 in synaptosomes). Depolarization of synaptosomes with 130 mM KCl increased the affinity of lifarizine almost 10 fold ($pIC_{50} = 7.86 \pm 0.25$). This suggests that lifarizine binds selectively to inactivated sodium channels which predominate both in the membrane preparation and in the depolarized synaptosomal preparation.

4 There was negligible [³H]-lifarizine and [³H]-BTX binding to solubilized sodium channels, although [³H]-STX binding was retained under these conditions.

5 The potencies of a series of compounds in displacing [³H]-lifarizine from rat cerebrocortical membranes correlated well with their affinities for inactivated sodium channels estimated from whole-cell voltage clamp studies in the mouse neuroblastoma cell line, N1E-115 ($r = 0.96$).

6 These results show that [³H]-lifarizine is a high affinity ligand for neuronal sodium channels which potently and selectively labels a site, allosterically linked to toxin binding site 2, associated with inactivated sodium channels.

Keywords: Lifarizine; sodium channels; BTX; rat cortex; N1E-115 cells; patch-clamp; RS-87476; sodium currents; ligand binding

Introduction

Lifarizine (RS-87476) is a diphenylpiperazine analogue that is neuroprotective in a number of animal models of focal and global cerebral ischaemia (Alps *et al.*, 1990; Kucharczyk *et al.*, 1991; Brown *et al.*, 1993; 1994). Furthermore, lifarizine has shown early promise in a small-scale clinical trial designed to assess its safety and tolerance in the treatment of acute ischaemic stroke (Squire *et al.*, 1994). This neuroprotective action is thought to be mediated via an interaction of the compound with CNS voltage-gated sodium channels. Thus, lifarizine potently inhibits the neurotoxic effects of the sodium channel activator, veratridine, on rat cerebrocortical neurones in culture (May *et al.*, 1995) and exerts a potent voltage-dependent inhibition of neuronal sodium currents, an action that is mediated by a preferential interaction of lifarizine with inactivated sodium channels (McGivern *et al.*, 1995). This latter action is very similar to that produced by a number of other agents which are believed to exert their therapeutic actions via an inhibition of sodium currents. For example, local anaesthetics inhibit sodium currents by an interaction with the inactivated state of the channel (for review, see Catterall, 1987).

It has been recognised for some time that there is a good correlation between the abilities of many agents to inhibit sodium currents and their abilities to displace the sodium channel activator, [³H]-batrachotoxin-A 20- α -benzoate (BTX), from its binding site (Postma & Catterall, 1984). BTX and other toxins have been used to probe sodium channel structure and func-

tion. A number of toxin binding sites have been defined for the sodium channel: tetrodotoxin (TTX) and saxitoxin (STX) bind to site 1 to inhibit sodium flux through the channel (Catterall, 1981); BTX, veratridine and aconitine bind to site 2 to induce persistent activation of the channel (Catterall, 1981; Hille *et al.*, 1987); the α -scorpion toxins and sea anemone toxins interact at site 3 to slow inactivation and potentiate the actions of toxins at site 2 (Lazdunski *et al.*, 1986); β -scorpion toxins bind to site 4 to induce a hyperpolarizing shift of the voltage-dependence of channel activation (Couraud *et al.*, 1982); the brevetoxins and the ciguatoxins bind to site 5, shift the voltage-dependence of channel activation and enhance the binding of toxins at sites 2 and 4 (Catterall & Risk, 1981; Poli *et al.*, 1986); pyrethroids and DDT act via a sixth unique site to cause persistent activation and also greatly enhance the binding of toxins to site 2 (Lombet *et al.*, 1988).

The local anaesthetic, tetracaine (Reith *et al.*, 1987), the putative local anaesthetic, PD85,639 (Thomsen *et al.*, 1993) and the anticonvulsant agent, phenytoin (Francis & Burnham, 1992), have been radiolabelled and used as probes to determine if they bind to novel sites on the voltage-gated sodium channel specific for the different therapeutic agents. Both classes of compounds allosterically interact with toxin site 2, although evidence suggests that local anaesthetic and anticonvulsant drugs do not bind to the same site on the sodium channel, as anticonvulsants (100 μ M) do not compete for [³H]-PD85,639 binding (Thomsen *et al.*, 1993) and local anaesthetics have very low affinity for [³H]-phenytoin binding (Francis & Burnham, 1992). Recently the location of the anaesthetic receptor site in the pore of the sodium channel has been defined (Ragsdale *et*

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al., 1994). In the present study we have characterized the binding of the cerebral anti-ischaemic agent, $[^3\text{H}]$ -lifarizine, to rat brain sodium channels and have investigated the functional relevance of the binding site in electrophysiological experiments in voltage-clamped neuroblastoma cells.

Methods

$[^3\text{H}]$ -lifarizine binding to rat cerebrocortical membranes

Male Sprague-Dawley rats (180–200 g) were decapitated and the cerebral cortex separated from other brain regions over ice. Membranes were prepared by homogenization in 25 volumes of ice-cold 50 mM Tris HCl (pH 7.4 at 25°C) with a Polytron PT 10 tissue disruptor. The homogenate was centrifuged at 48000 g for 15 min at 4°C. The pellet was resuspended in the same buffer and washed 3 times by resuspension and centrifugation. The final pellet was resuspended in Tris HCl buffer at an approximate protein level of 3 mg ml⁻¹, snap frozen in liquid N₂ and stored at -80°C until required. Binding assays were initiated by the addition of membrane protein (150–200 µg) to 50 mM Tris HCl (pH 7.4) containing 0.01% bovine serum albumin (BSA), 1 nM $[^3\text{H}]$ -lifarizine and various concentrations of test drugs (500 µl final assay volume). Non-specific binding was determined in the presence of 1 µM lifarizine or 3 µM flunarizine. Reactions were incubated for 90 min at 25°C and bound ligand was separated from free by vacuum filtration through Whatman GF/B filters presoaked for 30 min in assay buffer containing 0.01% polyethylenimine. The filters were washed with 3 × 5 ml assay buffer and bound ligand was estimated by liquid scintillation spectrometry. Protein content was determined with protein assay kits (Pierce BSA) using BSA as the protein standard.

Initial experiments showed that $[^3\text{H}]$ -lifarizine binding was linear up to 450 µg membrane protein and optimal between pH 6.0 and pH 7.8. Subsequently all assays were carried out with 150–200 µg membrane protein in 50 mM Tris HCl pH 7.4 containing 0.01% bovine serum albumin (BSA) to reduce non-specific binding. Under these conditions equilibrium was reached after 75 min with typical total binding values at 1 nM $[^3\text{H}]$ -lifarizine of 2500–3000 d.p.m. and 65–70% specific binding.

$[^3\text{H}]$ -BTX binding to rat cerebrocortical synaptosomes

Cerebral cortices from male Sprague-Dawley rats were roughly chopped and homogenized in a glass-Teflon hand-held homogenizer in approximately 10 volumes of ice-cold 0.32 M sucrose, 5 mM K₂HPO₄ (pH 7.4 at 4°C). The homogenate was centrifuged at 1000 g for 10 min, the pellet discarded and the supernatant centrifuged at 20000 g for 15 min. The resulting pellet was washed by resuspension in an equal volume of sucrose buffer and recentrifuged. The final pellet was suspended in a sodium-free assay buffer containing 50 mM HEPES, 5.4 mM KCl, 0.8 mM MgSO₄, 5.5 mM glucose and 130 mM choline chloride (pH 7.4 at 25°C). Binding assays were initiated by the addition of 150–200 µg synaptosomal protein to an assay buffer containing 1 µM TTX, 50 µg scorpion venom (*Leiurus quinquestriatus*), 5 nM $[^3\text{H}]$ -BTX and various concentrations of test drugs (500 µl final volume). Non-specific binding was determined in the presence of 0.3 mM veratridine. Reactions were incubated for 90 min at 25°C and bound ligand was separated from free by vacuum filtration through GF/B filters. The filters were washed with 2 × 5 ml buffer (5 mM HEPES, 1.8 mM CaCl₂, 0.8 mM MgSO₄, 130 mM choline chloride, 0.01% BSA; pH 7.4 at 25°C) and bound ligand was estimated by liquid scintillation spectrometry.

$[^3\text{H}]$ -lifarizine binding to rat cerebrocortical synaptosomes

To address the effects of depolarization on specific $[^3\text{H}]$ -lifarizine binding to cerebrocortical synaptosomes, experi-

ments were performed as described above for $[^3\text{H}]$ -BTX binding to rat cortical synaptosomes or substituting KCl (130 mM) for choline chloride in the assay buffer to produce a depolarization of the preparation.

$[^3\text{H}]$ -STX binding to rat cerebrocortical membranes

Cortical membranes were prepared as for the $[^3\text{H}]$ -lifarizine binding assay (see above). Membrane protein (200–400 µg) was incubated with 1 nM $[^3\text{H}]$ -STX in 500 µl assay buffer (50 mM Tris HCl, 0.01% BSA; pH 7.4) for 60 min at 25°C. Non-specific binding was determined in the presence of 1 µM TTX. Bound ligand was separated as described for $[^3\text{H}]$ -lifarizine binding.

Solubilization and reconstitution into phospholipid vesicles

Membranes were prepared from 2 rat cortices (see above) in ice-cold 50 mM Tris HCl buffer (pH 7.4 at 25°C) containing 1 mM EGTA and the protease inhibitors, phenyl methyl sulphonyl fluoride (PMSF; 0.1 mM), iodoacetamide (1 mM), phenanthroline (1 mM) and pepstatin A (1 µM). Membrane protein (40–50 mg) was suspended in 20 ml 10 mM HEPES (pH 7.4) containing protease inhibitors, 100 mM KCl, phosphatidyl choline (0.25% w/v) and detergent (2.5% Triton X-100, 0.3% sodium cholate or 1% β-octyl glucoside). The mixture was stirred on ice for 15 min before centrifugation at 120000 g for 75 min at 4°C. Solubilized sodium channels were reconstituted into phosphatidylcholine: phosphatidylethanolamine (65%:35%) vesicles as previously described (Feller et al., 1985).

Binding of $[^3\text{H}]$ -STX, $[^3\text{H}]$ -BTX or $[^3\text{H}]$ -lifarizine (all at 1 nM) was carried out at 4°C for 15 min. Non-specific binding was determined with 1 µM TTX for $[^3\text{H}]$ -STX binding, 0.3 mM veratridine for $[^3\text{H}]$ -BTX binding and 1 µM lifarizine for $[^3\text{H}]$ -lifarizine binding. Bound ligand was separated by filtration through GF/B filters which had been previously soaked overnight in 10% polyethylenimine in ice-cold solubilization buffer. Filters were washed twice with Tris HCl containing 0.01% BSA (pH 7.4 at 4°C) and bound ligand was estimated by liquid scintillation spectrometry.

Analysis of binding data

The radioligand displacement curves were analysed graphically to estimate the IC₅₀ (concentration of inhibitor displacing 50% of specifically bound radioligand), using a non-linear least squares programme specially designed for the interpretation of sigmoidal concentration curves in terms of total and non-specific binding as well as inhibition constants and curve steepness. Equilibrium binding parameters (K_d and B_{max}) for $[^3\text{H}]$ -lifarizine and $[^3\text{H}]$ -BTX were obtained from competition experiments with various concentrations of unlabelled lifarizine and BTX (Deblasi et al., 1989). Determination of dissociation kinetics was carried out at equilibrium by the addition of excess unlabelled drug or by dilution of the drug-receptor complex in 100 fold volume of assay buffer. The rate of radioligand dissociation was determined from a series of timed filtrations of the samples. The dissociation rate constant (K_{-1}) was calculated from the slope of the plot $\ln(B_t/B_0)$ versus time, where B_0 represents binding at time 0 (equilibrium) and B_t the binding at time t .

Electrophysiology

Voltage-dependent sodium currents were recorded from undifferentiated mouse neuroblastoma N1E-115 cells by conventional whole-cell voltage clamp techniques at room temperature (Hamill et al., 1981). Cells were obtained from the European Collection of Animal Cell Cultures and used between passages 20–45. The following solutions were used to isolate sodium currents (values in mM): pipette – CsF 120, tetraethylammonium

Cl 10, NaCl 10, CaCl_2 1, MgCl_2 1, EGTA 11, HEPES (free acid) 10, adjusted to pH 7.3 with CsOH; extracellular – NaCl 145, KCl 3, CaCl_2 1, MgCl_2 1, CdCl_2 0.5, HEPES (free acid) 10, D-glucose 5, adjusted to pH 7.3 with NaOH.

Cells were clamped at a holding potential of -100 mV and control sodium currents were evoked by 10 ms depolarizing steps to 0 mV. Following establishment of a stable current, cells were exposed to a single concentration of drug for 10 min. The membrane potential was then clamped to a conditioning potential of 0 mV for 20 s, after which it was returned to -100 mV and a series of test depolarizations to 0 mV were made to probe recovery from voltage-dependent block. The first test depolarization was elicited 2 s after the conditioning depolarization, with subsequent test depolarizations being evoked at a frequency of 0.2 Hz. Recovery from block followed an exponential time course which was fitted to the following function: relative $I_{\text{Na}} = 1 - A \cdot \exp(-t/\tau)$, where t is time (s), τ is the time constant of the exponential recovery (s) and $A = 1 - y$ intercept value (i.e. the extrapolated current amplitude immediately after the 20 s conditioning depolarization normalised to the pre-conditioning control current amplitude). The apparent dissociation constant (apparent K_b) of compounds for inactivated sodium channels was calculated from the following relation:

$$\text{apparent } K_b = \{(1/A) - 1\} \cdot [b] \quad (1)$$

where $[b]$ is the concentration of drug applied. The drug concentrations used were determined empirically and were sufficient to inhibit the first post-conditioning test pulse by 50–90% (see Results for individual drug concentrations). Eq. 1 was obtained by rearrangement of the logistic equation which states that $E = E_{\text{max}} \cdot 1/(1 + K_b/[b])$, where E is the effect produced by drug b , and E_{max} is the maximum effect obtained. In the context of the present experiments, E_{max} is equal to 1 (i.e. complete inhibition of sodium current immediately after the conditioning depolarization), and E is equivalent to the term A in Eq. 1. Note that Eq. 1 does not incorporate a Hill coefficient, and therefore a bimolecular 1:1 reaction between the drug and its receptor on the inactivated state of the channel is assumed in the K_b estimation. This limitation arises from the use of a single concentration of drug and is explicitly acknowledged in the term 'apparent K_b '. Only those cells in which a mono-exponential recovery from voltage-dependent block was observed were included in the data analysis.

Statistical analysis

Data are expressed as the mean $\pm$ s.e.mean. Significant differences ($P < 0.05$) were calculated by Student's t test.

Figure 1 Dissociation of $[^3\text{H}]$ -lifarizine from rat cerebrocortical membranes. The graph represents a single experiment (performed in triplicate). In this example, dissociation, initiated by the addition of $1 \mu\text{M}$ lifarizine, was rapid ($t_{1/2} = 6.5$ min; $K_{-1} = 0.1068 \text{ min}^{-1}$). Similar findings were obtained in two other experiments (see Results for mean values).

Drugs and reagents Reagents used were of the highest analytical grade available. Lifarizine (piperazine 1-[diphenyl-methyl]-4-[(2-(4-methyl-phenyl)-5-methyl imidazol-4-yl)methyl]-trihydrochloride, KB-2796 (1-[bis(4-fluorophenyl) methyl]-4-(2,3,4-trimethoxybenzyl) piperazine-2HCl) and PD85,639 (α -(phenyl)- N -[3-(2,6-dimethyl-1-piperizyl)- α -propyl]benzeneacetamide) were synthesized by the Recherche Syntex France or the Institute of Organic Chemistry, Syntex, Palo Alto, CA, U.S.A.; $[^3\text{H}]$ -lifarizine (17 Ci mmol^{-1}) was synthesized by Dr H. Parnes (Institute of Organic Chemistry, Syntex, Palo Alto, CA, U.S.A.); $[^3\text{H}]$ -batrachotoxinin-A 20- α -benzoate (56 Ci mmol^{-1} ; Du Pont, U.K.); $[^3\text{H}]$ -saxitoxin (39 Ci mmol^{-1} ; Amersham, U.K.); unlabelled batrachotoxin was kindly donated by Dr J Daly, NIH Bethesda; lidoflazine (Janssen); phenytoin sodium (Parke-Davis); dizocilpine (Research Biochemicals Inc.); brevetoxin (CalBiochem); aconitine, veratridine, lignocaine HCl, flunarizine, tetrodotoxin and scorpion venom (*Leiurus quinquestriatus*, Sigma V5251) (Sigma U.K.).

Results

$[^3\text{H}]$ -lifarizine binding in rat cerebrocortical membranes

Bound $[^3\text{H}]$ -lifarizine was potentially displaced from cerebrocortical membranes by unlabelled lifarizine ($\text{pIC}_{50} = 7.99 \pm 0.09$, $n = 6$) with a Hill slope not significantly different from unity ($n_H = 0.87 \pm 0.08$, Student's two tailed t test). Figure 1 shows that $[^3\text{H}]$ -lifarizine binding was rapidly reversed by the addition of $1 \mu\text{M}$ unlabelled lifarizine ($K_{-1} = 0.1002 \pm 0.0128 \text{ min}^{-1}$; $n = 3$). Saturation studies revealed that $[^3\text{H}]$ -lifarizine labelled a single class of sites in rat cerebrocortical membranes ($K_d = 10.7 \pm 2.9 \text{ nM}$; $B_{\text{max}} = 5.10 \pm 1.43 \text{ pmol mg}^{-1} \text{ protein}$; $n = 5$; Figure 2).

Correlation of $[^3\text{H}]$ -lifarizine and $[^3\text{H}]$ -BTX binding

Cold lifarizine displaced $[^3\text{H}]$ -BTX binding with moderate affinity ($\text{pIC}_{50} 7.31 \pm 0.24$), suggesting an interaction with toxin site 2. Table 1 shows the affinities of a series of compounds for $[^3\text{H}]$ -lifarizine binding to rat cerebrocortical membranes and $[^3\text{H}]$ -BTX binding to rat cerebrocortical synaptosomes. There was a good correlation between the abilities of compounds to displace $[^3\text{H}]$ -lifarizine and $[^3\text{H}]$ -BTX ($r = 0.94$; Table 1 and Figure 3).

Influence of neurotoxins on $[^3\text{H}]$ -lifarizine binding

Neurotoxins that interact with distinct toxin sites on the sodium channel were evaluated for their effect on $[^3\text{H}]$ -lifarizine

Figure 2 Saturation analysis of 1 nM $[^3\text{H}]$ -lifarizine binding to rat cerebrocortical membranes. The graph represents a single experiment (performed in duplicate). The equilibrium binding constants in this example were: $K_d = 7.23 \text{ nM}$ and $B_{\text{max}} = 4.92 \text{ pmol mg}^{-1} \text{ protein}$. Similar findings were obtained in four other experiments (see Results for mean values).

Table 1 Affinity values for $[^3\text{H}]$ -BTX binding to rat cerebrocortical synaptosomes and $[^3\text{H}]$ -lifarizine binding to rat cerebrocortical membranes

	pIC_{50} $[^3\text{H}]$ -BTX	n_H	pK_i $[^3\text{H}]$ -lifarizine	n_H
Aconitine	6.29 ± 0.13	1.04 ± 0.09	5.47 ± 0.20	1.02 ± 0.09
Veratridine	5.44 ± 0.20	1.06 ± 0.12	4.67 ± 0.17	—
Phenytoin	4.69 ± 0.13	—	4.90 ± 0.12	—
Lignocaine	4.26 ± 0.21	—	4.48 ± 0.41	0.98 ± 0.09
PD85,639	6.59 ± 0.06	1.00 ± 0.11	6.43 ± 0.32	0.76 ± 0.10
Lidoflazine	7.25 ± 0.12	1.27 ± 0.25	7.52 ± 0.20	0.86 ± 0.11
Flunarizine	7.43 ± 0.21	0.99 ± 0.06	7.55 ± 0.15	0.89 ± 0.04
Lifarizine	7.31 ± 0.24	0.97 ± 0.07	8.03 ± 0.09	0.87 ± 0.08
KB-2796	7.34 ± 0.10	0.72 ± 0.15	7.70 ± 0.07	1.17 ± 0.27
Dizocilpine	5.32 ± 0.06	0.78 ± 0.09	5.31 ± 0.08	1.09 ± 0.16

Rat cerebrocortical synaptosomes ($[^3\text{H}]$ -BTX) or membranes ($[^3\text{H}]$ -lifarizine) were incubated with radioligand in the presence or absence of a range of concentrations of competing drug as described in Methods. The data represent the mean ± s.e.mean of at least 3 determinations performed in duplicate. The $[^3\text{H}]$ -BTX affinities are presented as pIC_{50} values corrected for ligand concentration.

Figure 3 Correlation of binding affinity for $[^3\text{H}]$ -lifarizine and $[^3\text{H}]$ -BTX in rat cerebrocortical preparations. The data points are taken from Table 1. The solid line represents the line of best fit ($r = 0.9$, slope = 1.1).**Table 2** Effect of lifarizine on equilibrium binding of $[^3\text{H}]$ -BTX to rat cerebrocortical synaptosomes

	n	K_d (nM)	B_{max} (pmol mg ⁻¹)
Control	8	509 ± 81	10.12 ± 1.99
Lifarizine 10 nM	3	364 ± 71	7.29 ± 0.94
30 nM	3	651 ± 293	6.13 ± 2.54
100 nM	3	856 ± 113*	4.69 ± 0.31*

Rat cerebrocortical synaptosomes were incubated with 5 nM $[^3\text{H}]$ -BTX and unlabelled BTX (0.3 nM–10 μM) in the presence or absence of lifarizine, as described in Methods. The data represent the mean ± s.e.mean of three determinations (performed in duplicate).

* $P < 0.05$, Student's t test.

binding (data not shown). The site 1 toxin, TTX (1 μM , $n = 3$), had no effect on $[^3\text{H}]$ -lifarizine binding. Moreover, lifarizine, at concentrations up to 10 μM ($n = 3$), had no effect on $[^3\text{H}]$ -STX binding to rat cerebrocortical membranes. α -Scorpion venom (up to 150 μg) and brevetoxin (10 μM) were similarly without effect on $[^3\text{H}]$ -lifarizine binding. These data suggest that lifarizine does not interact with and is not influenced by toxin sites 1, 3 or 5.

Mechanism of interaction of lifarizine with the $[^3\text{H}]$ -BTX binding site

The mechanism of the interaction of lifarizine with toxin site 2 was explored further by studying the effect of lifarizine on kinetic and equilibrium properties of $[^3\text{H}]$ -BTX binding.

Saturation analysis of $[^3\text{H}]$ -BTX binding revealed that this ligand bound to cerebrocortical synaptosomes in the presence of α -scorpion venom with $K_d = 509 \pm 81$ nM and $B_{max} = 10.12 \pm 1.99$ pmol mg⁻¹ protein. Co-incubation with lifarizine (10, 30 or 100 nM) produced a concentration-dependent decrease in the affinity and density of sites labelled by $[^3\text{H}]$ -BTX, both of which were statistically significant at 100 nM lifarizine (Table 2). These data indicated that the interaction of lifarizine with toxin site 2 is not competitive. In kinetic studies, the dissociation of $[^3\text{H}]$ -BTX, initiated by an excess of the competitive inhibitor, veratridine (0.3 mM), was slow with $K_{-1} = 0.00945 \pm 0.00096$ min⁻¹ ($n = 5$; Figure 4). In the presence of a saturating concentration (1 μM) of lifarizine there was a small but significant increase in the dissociation rate of $[^3\text{H}]$ -BTX ($K_{-1} = 0.0139 \pm 0.0018$ min⁻¹, $n = 5$, $P < 0.05$). When $[^3\text{H}]$ -BTX was dissociated by dilution of the bound ligand in 100 fold volume of incubation buffer, $[^3\text{H}]$ -BTX dissociated very slowly, with less than 15% of the specific binding dissociated after 3 h (data not shown) at room temperature. Addition of lifarizine (1 μM) to the dilution buffer markedly accelerated the dissociation of $[^3\text{H}]$ -BTX with more than 75% dissociated after 2 h and $K_{-1} = 0.025$ min⁻¹ ($n = 2$; 0.0234 and 0.0265 min⁻¹).

Effect of depolarization

Electrophysiological experiments have demonstrated that the inhibitory effect of lifarizine on whole-cell sodium currents is markedly enhanced by membrane depolarization (McGivern *et al.*, 1995). We therefore examined the effect of KCl-induced depolarization on $[^3\text{H}]$ -lifarizine binding to cerebrocortical synaptosomes. $[^3\text{H}]$ -lifarizine binding to synaptosomes was of lower affinity than in membranes: pIC_{50} for unlabelled lifarizine = 7.99 ± 0.09 in membranes and 6.68 ± 0.14 in synaptosomes. However, depolarization of synaptosomes with 130 mM KCl increased the affinity of lifarizine almost 10 fold ($pIC_{50} = 7.86 \pm 0.25$). Thus, $[^3\text{H}]$ -lifarizine binding appears to be voltage-sensitive.

Solubilization and reconstitution studies

Rat cerebrocortical membranes were solubilized with Triton X-100, as described by Hartshorne & Catterall (1984). Phosphatidylcholine was included in the solubilization buffer because the presence of lipid has been found to increase greatly the stability of the α -subunit (Agnew & Raftery, 1979; Hartshorne & Catterall, 1981). Although the soluble preparation

retained $[^3\text{H}]$ -STX binding, $[^3\text{H}]$ -BTX and $[^3\text{H}]$ -lifarizine binding were negligible under these conditions (Table 3). Solubilization of the membranes in other detergents (sodium cholate and β -octyl glucoside) also failed to produce consistently preparations retaining workable amounts of specific $[^3\text{H}]$ -lifarizine and $[^3\text{H}]$ -BTX binding (Table 3). When cholate-solubilized membranes were reconstituted into phosphatidylcholine: phosphatidylethanolamine vesicles, specific $[^3\text{H}]$ -lifarizine binding was increased from $18 \pm 6\%$ ($n=3$) to 53% ($n=2$; 48% and 58%).

Figure 4 Dissociation of $[^3\text{H}]$ -BTX from rat cerebrocortical synaptosomes. The two graphs are from separate experiments (performed in triplicate). Similar data were obtained in additional experiments (see Results for mean values). Assays were brought to equilibrium (90 min at 25°C) and dissociation was initiated either by addition of 0.3 mM veratridine (a) or by dilution of 100 fold volume of assay buffer (b). In (a), veratridine-induced dissociation was slow under control conditions ($\circ$ $t_{1/2}=90$ min and $K_{-1}=0.00767$ min^{-1}) but was accelerated by $1 \mu\text{M}$ lifarizine ($\bullet$ $t_{1/2}=71$ min and $K_{-1}=0.00970$ min^{-1}). In (b), dilution-induced dissociation was slow (control $\circ$ $t_{1/2} > 500$ min) but was greatly accelerated by $1 \mu\text{M}$ lifarizine ($\bullet$ $t_{1/2}=52$ min and $K_{-1}=0.0234$ min^{-1}).

Table 3 Specific binding of $[^3\text{H}]$ -STX, $[^3\text{H}]$ -BTX and $[^3\text{H}]$ -lifarizine to soluble rat cerebrocortical preparation

Detergent	Ligand		
	$[^3\text{H}]$ -STX	$[^3\text{H}]$ -BTX	$[^3\text{H}]$ -lifarizine
Triton X-100 (2.5%)	95 ± 1	9 ± 4	0
Cholate (0.3%)	76 ± 8	31 ± 15	18 ± 6
β -Octyl glucoside (1%)	71 ± 9	0	0

Binding of 1 nM ligand was determined with three different detergents at the concentrations indicated as described in Methods. The data represent the % specific binding of 1 nM ^3H -labelled ligand in each of the solubilized preparations and shown as the mean $\pm$ s.e. mean of three determinations (performed in duplicate).

Functional relevance of the $[^3\text{H}]$ -lifarizine binding site

The functional relevance of $[^3\text{H}]$ -lifarizine binding was investigated by correlating the potency with which a series of compounds displaced $[^3\text{H}]$ -lifarizine from rat cerebrocortical membranes with their apparent affinities for inactivated sodium channels estimated from whole-cell voltage clamp experiments in the mouse neuroblastoma cell line, N1E-115. The following compounds were included in the correlation: lifarizine ($1 \mu\text{M}$; $n=4$); flunarizine ($1 \mu\text{M}$; $n=4$); KB-2796 ($3 \mu\text{M}$; $n=3$); PD85,639 ($3 \mu\text{M}$; $n=3$); phenytoin (50 and 100 μM ; $n=4$ at each concentration); lignocaine (30, 100 and 300 μM ; $n=1$ at each concentration).

An example of the estimation of apparent K_b for lifarizine is illustrated in Figure 5. This shows the time-course of recovery from voltage-dependent block following a 20 s conditioning depolarization in the presence of $1 \mu\text{M}$ lifarizine. A mono-exponential fit to the data points yielded a y-intercept value of 0.145 (i.e. $A = 0.855$), giving an apparent K_b of $0.17 \mu\text{M}$.

A correlation of apparent pK_b versus pK_i for displacement of bound $[^3\text{H}]$ -lifarizine for all compounds tested is shown in

Figure 5 Recovery of sodium channels from voltage-dependent block. This graph shows recovery of sodium channels from voltage-dependent block in a single N1E-115 cell, exposed to $1 \mu\text{M}$ lifarizine. In this example, the data points are well-described by a mono-exponential function which gives an estimate for A of 0.855. The calculated apparent affinity (K_b) for the inactivated state of sodium channel is $0.17 \mu\text{M}$.

Figure 6 Correlation of apparent affinity for inactivated sodium channels with binding affinity for the $[^3\text{H}]$ -lifarizine site. This graph shows the correlation of apparent affinity (pK_b) for the inactivated state of the sodium channel with affinity (pK_i) for the $[^3\text{H}]$ -lifarizine site for a series of compounds ($r = 0.96$).

Figure 6. The correlation obtained ($r=0.96$) provides strong evidence that affinity for the $[^3\text{H}]$ -lifarizine binding site is closely related to affinity for inactivated sodium channels.

Discussion

The present study demonstrates that $[^3\text{H}]$ -lifarizine binds saturably and reversibly to a single class of high affinity (10.7 nM) sites on rat cerebrocortical membranes. The density of sites labelled by $[^3\text{H}]$ -lifarizine was 5.1 pmol mg^{-1} protein, which is similar to the reported density of sodium channels in rat brain as determined by $[^3\text{H}]$ -STX binding (4.9 pmol mg^{-1} protein; Catterall *et al.*, 1979), and to the density of sites labelled by other radioligands known to interact with the sodium channel ($[^3\text{H}]$ -PD85,639: 5.2 pmol mg^{-1} protein, Thomsen *et al.*, 1993; $[^3\text{H}]$ -phenytoin: 4.5 pmol mg^{-1} protein, Francis & Burnham, 1992; $[^3\text{H}]$ -tetracaine: 5.2 pmol mg^{-1} protein, Reith *et al.*, 1987) and suggests a 1:1 stoichiometry for all these binding sites on the sodium channel. These results therefore provided initial circumstantial evidence that the site labelled by $[^3\text{H}]$ -lifarizine is located on the sodium channel. Further support for this conclusion was provided by the finding that lifarizine displaced the site 2 sodium channel toxin, $[^3\text{H}]$ -BTX, from rat cerebrocortical synaptosomes. Furthermore, there was a very good correlation between the ability of a series of compounds to displace $[^3\text{H}]$ -BTX and their ability to displace $[^3\text{H}]$ -lifarizine ($r=0.9$), implying that the sites labelled by the two ligands are in some way linked. However, unlike $[^3\text{H}]$ -BTX binding, $[^3\text{H}]$ -lifarizine binding was not influenced by α -scorpion venom or the site 5 toxin, brevetoxin both of which potentiate $[^3\text{H}]$ -BTX binding, suggesting that the modes of interaction of the two radioligands with site 2 are dissimilar.

Lifarizine produced both a decrease in the affinity and the number of sites labelled by $[^3\text{H}]$ -BTX. This suggests that the interaction of lifarizine with site 2 was allosteric in nature. The interaction with site 2 was probed further by examining the effect of lifarizine on the dissociation rate of $[^3\text{H}]$ -BTX estimated by two methods: by initiating dissociation through addition of excess competitive agent (e.g. veratridine), or by diluting the $[^3\text{H}]$ -BTX/channel complex in 100 fold volume of assay buffer. In the first method, as $[^3\text{H}]$ -BTX dissociates its reassociation is prevented by the presence of excess veratridine. Any change in the dissociation rate that occurs in the presence of an additional compound (e.g. lifarizine) indicates that the compound is interacting at a site distinct from the $[^3\text{H}]$ -BTX binding site. In these experiments, a saturating concentration of lifarizine (1 μM) accelerated the veratridine-induced $[^3\text{H}]$ -BTX dissociation, suggesting that lifarizine interacts allosterically with toxin site 2. In the second method (diluting the $[^3\text{H}]$ -BTX/channel complex at equilibrium), reassociation is also prevented since the free concentration of $[^3\text{H}]$ -BTX is reduced 100 fold. With this method, lifarizine (1 μM) accelerated the dissociation of $[^3\text{H}]$ -BTX, again suggesting an allosteric interaction. However, in the second method the effect of lifarizine was more marked because the control rate of $[^3\text{H}]$ -BTX dissociation was much slower than in the first method. Indeed, dilution-induced $[^3\text{H}]$ -BTX dissociation was negligible under control conditions. In a non-cooperative system similar dissociation rates ought to be obtained with the two experimental procedures. Negative cooperativity could be one explanation as to why the veratridine-induced dissociation of $[^3\text{H}]$ -BTX was faster than the dilution-induced dissociation (in a negatively cooperative system, occupation of vacant sites by competing drug would decrease the overall affinity of the receptor population for radioligand and increase the rate of radioligand dissociation). An alternative explanation could be that veratridine does not act competitively at toxin site 2; however, there are data to suggest that it does (e.g. Catterall *et al.*, 1981). Further studies are required to elucidate which mechanism is operational here. Irrespective of which is operational, the data suggest that lifarizine acts allosterically to inhibit $[^3\text{H}]$ -BTX binding. These kinetic data support the results from the equilibrium binding studies.

Solubilization of the sodium channel with Triton X-100, under the conditions described by Hartshorne & Catterall (1984), did not affect the binding of $[^3\text{H}]$ -STX but abolished $[^3\text{H}]$ -BTX and $[^3\text{H}]$ -lifarizine binding. Subsequent attempts, with different detergents, also failed to produce a solubilized sodium channel which retained either $[^3\text{H}]$ -BTX or $[^3\text{H}]$ -lifarizine binding. These studies suggest that lifarizine, like BTX, requires the presence of phospholipids to maintain the channel in its native conformation. CNS sodium channels are composed of an α subunit and two β subunits ($\beta 1$ and $\beta 2$). The presence of the $\beta 1$ subunit has been shown, in reconstitution studies, to stabilize the functional state of purified brain sodium channels in detergent solution and to be required for $[^3\text{H}]$ -BTX binding (Isom *et al.*, 1994). Initial studies have shown that the binding of $[^3\text{H}]$ -lifarizine was greatly increased in reconstituted sodium channel preparations and in CHO-K1 cells expressing the α and $\beta 1$ subunits but not in cells expressing the α subunit only (N.P. Gillard & C.M. Brown, unpublished data). This lends further support to the idea that the channel needs to be in its native conformation for $[^3\text{H}]$ -lifarizine binding to occur.

The electrophysiological experiments were undertaken in an effort to determine the functional relevance of the $[^3\text{H}]$ -lifarizine binding site. Previous studies of the inhibitory effect of lifarizine on whole-cell sodium currents in N1E-115 cells have established that the inhibition is mediated by a preferential interaction of the compound with inactivated sodium channels (McGivern *et al.*, 1995). The present whole-cell voltage clamp experiments were designed to estimate the affinities of a series of sodium channel blocking agents for inactivated sodium channels in N1E-115 cells. Six compounds were examined and their apparent dissociation constants for inactivated channels ranged from 85 μM (lignocaine) to 0.17 μM (lifarizine). The apparent dissociation constants of the six compounds, as determined by an electrophysiological method, correlated remarkably well with their potencies in displacing $[^3\text{H}]$ -lifarizine from rat cerebrocortical membranes ($r=0.96$), strongly suggesting that $[^3\text{H}]$ -lifarizine labels a site on the channel associated with the inactivated state. This was further supported by the finding that the affinity of $[^3\text{H}]$ -lifarizine in rat cerebrocortical synaptosomes under depolarized conditions (130 mM K^+ , which completely depolarizes synaptosomes, e.g. Catterall *et al.*, 1981) was some 10 fold higher than under normal conditions. Indeed, the affinity of $[^3\text{H}]$ -lifarizine in depolarized synaptosomes was similar to its affinity in cerebrocortical membranes. The most likely explanation for these findings is that lifarizine binds selectively to inactivated sodium channels, since these will predominate both in the membrane preparation and in the depolarized synaptosomal preparation. The lower affinity of $[^3\text{H}]$ -lifarizine in non-depolarized synaptosomes may be due to a proportion, albeit unknown, of the sodium channels in this preparation being in the resting state, for which lifarizine has very low affinity (McGivern *et al.*, 1995).

Electrophysiological studies have demonstrated that lifarizine has negligible interaction with the open state of the sodium channel (McGivern *et al.*, 1995). In contrast, $[^3\text{H}]$ -BTX interacts preferentially with open sodium channels (Catterall, 1980). The possibility that $[^3\text{H}]$ -lifarizine and $[^3\text{H}]$ -BTX interact with different states of the channel is highlighted by the abilities of α -scorpion venom and brevetoxin to enhance $[^3\text{H}]$ -BTX binding by inducing an open (BTX-preferring) state of the channel, but not $[^3\text{H}]$ -lifarizine binding. Inhibition of $[^3\text{H}]$ -BTX binding by lifarizine may reflect a conformational change of the channel from the 'open' state, resulting in the channel being in a low affinity state for $[^3\text{H}]$ -BTX. The allosteric inhibition of $[^3\text{H}]$ -BTX binding may therefore be explained by an ability of lifarizine to stabilize the inactivated state of the sodium channel (as demonstrated in electrophysiological studies).

In conclusion, the present study has shown that $[^3\text{H}]$ -lifarizine binds with high affinity to a site allosterically linked to toxin binding site 2 on the CNS voltage-sensitive sodium channel. Taken together, the electrophysiological and ligand binding data suggest that this site is associated with an inactivated state of the channel.

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