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Ralitoline (CI-946) and CI-953 block sustained repetitive sodium action potentials in cultured mouse spinal cord neurons and displace batrachotoxinin A 20-α-benzoate binding in vitro

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Ralitoline and CI-953 are anticonvulsant compounds active in both maximal electroshock and kindling models of seizures with rodents. CI-953 (IC_{50} = 5 μM) and ralitoline (IC_{50} = 2 μM) both blocked sustained repetitive firing of sodium action potentials with effects on firing activity triggered by spontaneous excitatory postsynaptic potentials at higher concentrations. No effects on iontophoretic GABA and glutamate responses were noted. Both compounds inhibited the binding of tritiated batrachotoxinin A 20-α-benzoate ([^{3}H]BTX-b) to rat brain synaptosomes with apparent K_d values of 29 μM (CI-953) and 25 μM (ralitoline). Our results suggest that effects on voltage-dependent sodium channels may underlie the anticonvulsant action of these compounds.

INTRODUCTION

Ralitoline (CI-946) and CI-953 (Fig. 1) are structurally related compounds with efficacy in preclinical anticonvulsant models. Both compounds potently prevent maximal electroshock-induced seizures in rats and mice when given orally and they also raise afterdischarge thresholds in kindled rats. However, neither compound prevents clonic seizures from a variety of chemical convulsant agents in mice (bicuculline, pentylenetetrazol, strychnine, picrotoxin)[1,15,23]. This profile of anticonvulsant activity is similar to those of phenytoin and carbamazepine[7], standard anticonvulsant compounds used in the treatment of generalized tonic-clonic and partial seizures in man.

Both phenytoin and carbamazepine prevent

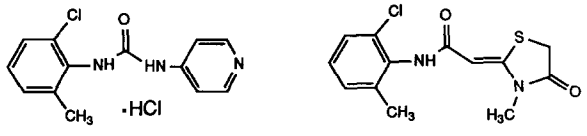

Fig. 1. Chemical structures of CI-953 and ralitoline.

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trains of rapidly repeated sodium action potentials in vitro from sustained depolarization[11,12] and there is now considerable evidence that they may exert their anticonvulsant effects by acting at voltage-sensitive sodium channels involved in the initiation and propagation of action potentials in the brain[8,10,14,17,20–22,24,27]. In addition, phenytoin and carbamazepine inhibit the binding of tritiated batrachotoxinin A 20-α-benzoate (BTX-b), a ligand for a specific site on voltage-dependent sodium channels that is associated with anticonvulsant and local anesthetic action[5,26,28]. The following studies were undertaken to determine if ralitoline and CI-953 share similar effects to those of phenytoin and carbamazepine on repetitive action potentials and on BTX-b binding. Preliminary results of some of these studies were presented in abstract form[23].

MATERIALS AND METHODS

Electrophysiological experiments

Cultures of fetal mouse spinal cord neurons were prepared by standard methods[18]. For electrophysiological experiments, cultures were placed on the heated stage of an inverted phase contrast microscope and intracellular impalements were obtained with high-resistance glass micropipettes (20–60 MΩ) filled with either 4 M potassium acetate or 3 M KCl. Membrane potential was recorded with a conventional high-impedance bridge amplifier and displayed on a rectilinear recorder and recorded on tape. The experimental medium was Dulbecco's phosphate-buffered saline (DPBS: NaCl 137 mM/KCl 3 mM/Na_2HPO_4 8 mM/KH_2PO_4 1.5 mM/$MgCl_2$ 1 mM (spontaneous activity experiments) or substituted with 10 mM $MgCl_2$ (repetitive firing and iontophoretic experiments)/$CaCl_2$ 1 mM/D-glucose 10 mM, pH 7.3–7.4).

Samples of CI-953 and ralitoline were obtained from Parke-Davis Research. Concentrations of ralitoline and CI-953 are expressed as the equivalent concentration of free base or acid and were dissolved into dimethyl sulfoxide (DMSO) and added to the bath to achieve the desired concentration. In each case, final concentration of DMSO was not greater than 0.1%. Previous control experiments with concentrations of DMSO up to 1.0% showed no change in sustained firing, GABA or glutamate responses (data not shown). The bathing medium was static and was maintained at temperatures between 35–37 °C.

Tests were performed to determine the effect of ralitoline and CI-953 on sustained repetitive firing of sodium action potentials, spontaneous neuronal activity and postsynaptic GABA and glutamate responses. Methods are identical to those of Rock et al.[19]. IC_{50} values for repetitive firing and spontaneous activity experiments were obtained by probit analysis and significant differences from control ($P \leq 0.05$) determined by a Wilcoxon rank-sum test. In iontophoretic experiments, significant difference from predrug application was determined by a 2-tailed Student's *t*-test.

Binding experiments

Batrachotoxinin was obtained from Dr. John Daly, Laboratory of Bioorganic Chemistry, NIDDK, NIH. Aconitine and veratridine were from Aldrich Chemical Company. [^{3}H]Batrachotoxinin A 20-α-benzoate ([^{3}H]BTX-b) was from New England Nuclear Corporation. The principal α-scorpion toxin from *Leiurus quinquestriatus* was purified by a modification of previously described procedures[3,25]. Synaptosomes were prepared as described previously[26]. Stock solutions of 10 mM ralitoline or CI-953 were made in ethanol, and diluted to give the final experimental concentrations of 1% ethanol. Control samples contained equivalent vehicle concentrations.

Specific binding of [^{3}H]BTX-b was measured essentially as described previously[6,26]. Prior to use, an aliquot of frozen synaptosomes was thawed at 36 °C for 5 min and then stored on ice. Binding reactions were initiated by mixing 25 μl of synaptosome suspension containing approximately 250 μg of synaptosomal protein with 175 μl of reaction mixture in standard binding medium (130 mM choline chloride/50 mM Hepes adjusted to pH 7.4 with Tris base/5.5 mM glucose/0.8 mM $MgSO_4$/5.4 mM KCl) containing 10 nM [^{3}H]BTX-b/1 μM tetrodotoxin/300 nM scorpion toxin/1 mg/ml bovine serum albumin and the indicated concentration of drugs. The samples were incubated at 36 °C for 30 min. Synaptosomes and bound [^{3}H]BTX-b were collected by rapid filtration on glass fiber filters

(Whatman GF/C) and washed 3 times with 3 ml of wash medium consisting of 163 mM choline chloride/5 mM Hepes-Tris (pH 7.4)/1.8 mM $CaCl_2$/0.8 mM $MgSO_4$/1 mg/ml bovine serum albumin. Bound [^{3}H]BTX-b was determined in a liquid scintillation counter. Nonspecific binding determined in the presence of 300 μM veratridine[6] was subtracted from all the results presented.

Synaptosomal protein concentrations were determined according to the method of Peterson[16]. The results presented represent pooled data from 3 or more experiments in which each concentration of drug was examined in duplicate. Error bars represent standard error of the mean. Values for apparent K_d were estimated by probit analysis of the binding data and were corrected for small effects of the concentration of [^{3}H]BTX-b (10 nM) and sodium channels (5 nM) in comparison to the K_d for BTX-b (82 nM[6]).

RESULTS

In control conditions, mouse spinal cord neurons respond to increasing amplitude 450 msec depolarizing current steps with trains of sodium action potentials that increase in frequency with larger amplitude current steps (Fig. 2, left panel). CI-953 (38 μM, Fig. 2, right panel) blocked the ability of

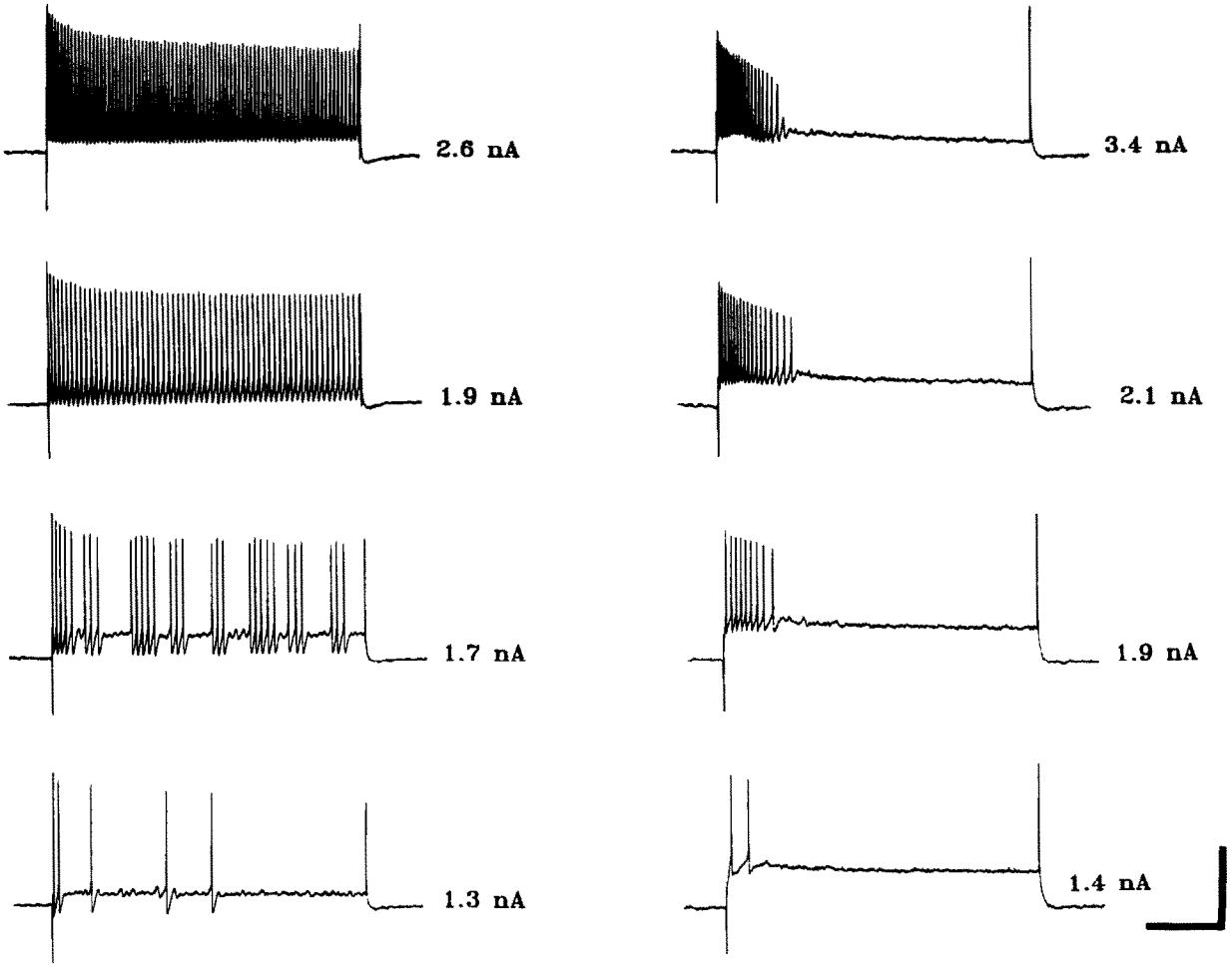

Fig. 2. CI-953 pretreatment prevents rapid sustained repetitive action potentials from depolarizing current steps in cultured spinal cord neurons. Responses in the left-hand series of traces are representative of untreated cultures, and those on the right are from another neuron representative of those treated with 38 μM CI-953. Note that high-frequency firing was not sustained after treatment with CI-953. Calibration bars are 50 mV and 100 msec.

cells to fire sustained high frequency action potentials. Both CI-953 (Fig. 3A; IC_{50} = 5 μM) and ralitoline (Fig. 3B; IC_{50} = 2 μM) reduced the percentage of cells that exhibited sustained repetitive firing of sodium action potentials in a concentration-dependent manner. Higher concentrations of both compounds were required to reduce spontaneous synaptically driven action potentials (Fig. 3).

High concentrations of CI-953 did not affect postsynaptic GABA or glutamate responses while diazepam reversibly enhanced GABA responses and phenobarbital reversibly reduced glutamate responses in agreement with published reports

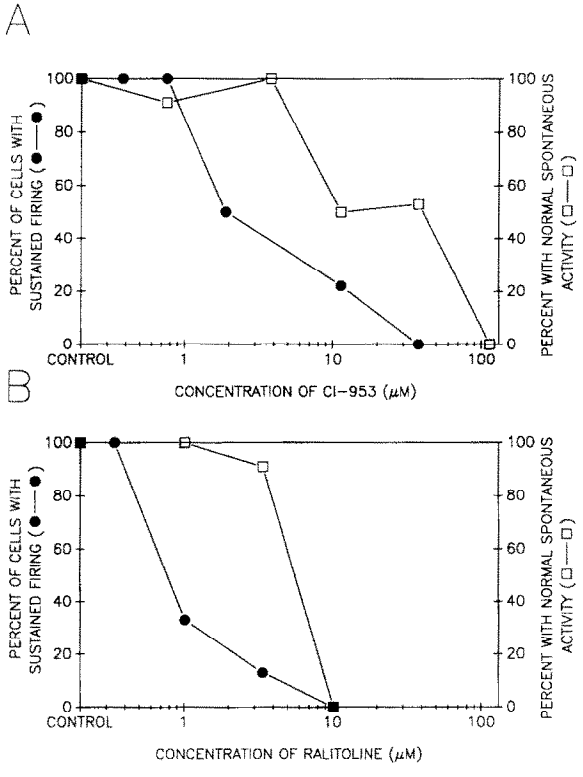

Fig. 3. Both CI-953 (A) and ralitoline (B) inhibit sustained high-frequency action potential firing in a dose-dependent manner. Closed symbols represent the percentage of cells with sustained firing at a variety of different drug concentrations. Spontaneous action potential activity (elicited by spontaneous postsynaptic potentials, not shown) was also reduced at somewhat higher concentrations (open squares). If an intracellular recording showed spontaneous activity that was markedly reduced from that seen in a large number of recordings in control medium, it was graded as reduced and the percentage of recordings with reduced activity is shown in the figure. Each data point represents recordings from 4 to 15 neurons.

TABLE I

Effect of ralitoline (CI-946) and CI-953 on iontophoretic GABA and glutamate responses

Drug	*Test concentration*	*% Control response (mean ± S.D.)*	*Unbound plasma level*
GABA			
CI-953	76 μM	99 ± 0.5 (n=5)	4.3 μM
Ralitoline	100 μM	100 ± 2.4 (n=4)	1.1 μM
Diazepam	100 nM	181 ± 21.7 (n=5)*	
Glutamate			
CI-953	76 μM	98 ± 1.8 (n=5)	4.3 μM
Phenobarbital	200 μM	84 ± 3.0 (n=5)*	

* $P \leq 0.05$ Student's t-test.

(Table I). Ralitoline did not affect postsynaptic GABA responses. No changes in membrane voltage or conductance were measured when high concentrations (76 μM of CI-953 or 100 μM of ralitoline) were applied to the cells during the iontophoretic experiments.

In radioligand binding experiments, the concentration dependences of inhibition of specific binding of [^{3}H]BTX-b by CI-953 and ralitoline are illustrated in Fig. 4. CI-953 inhibited binding with an apparent K_d value of 29 μM, and the data were fit by a binding curve for a single class of sites with apparent Hill coefficient of 1.08. Similarly ralitoline had a K_d value of 25 μM and Hill coefficient of 1.16; these values are shown in Table III in comparison to published values[26] for phenytoin and carbamazepine.

DISCUSSION

Concentrations of drug in plasma were calculated from pharmacokinetic data for doses (ED_{50}) that protect rats or mice from maximal electroshock tonic extensor seizures[7]. These data are shown for CI-953, ralitoline, phenytoin and carbamazepine in Tables I and II. The values calculated for unbound drug in plasma at the ED_{50} dose are 4.3 μM for CI-953 and 1.1 μM for ralitoline (H. Bockbrader and A. von Hodenberg, unpublished observations). The present experiments show that ralitoline and CI-953 both affect high-frequency firing of sodium action potentials at these concen-

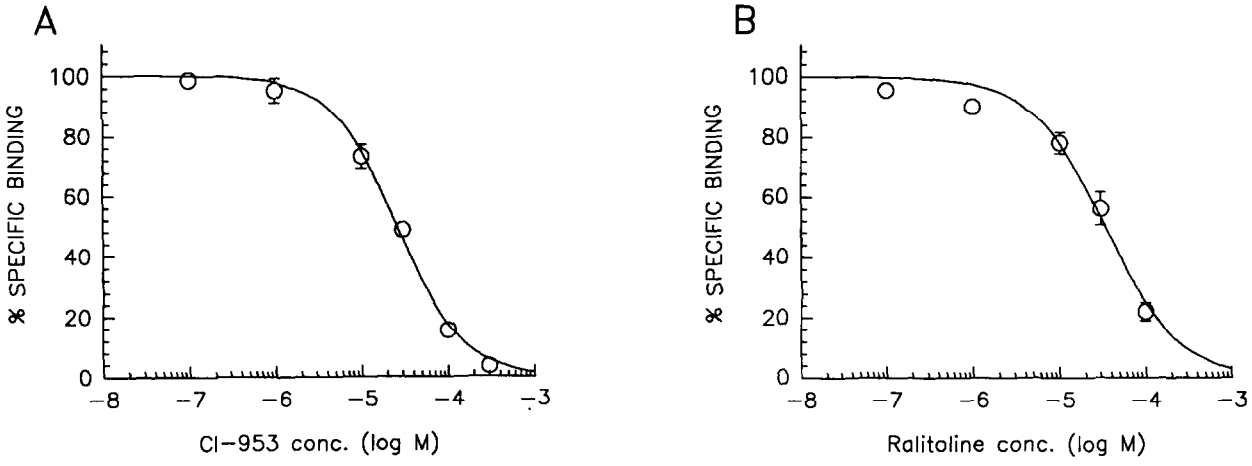

Fig. 4. Effects of CI-953 and CI-946 on BTX-b binding to sodium channels in rat brain synaptosomes. Symbols represent the specific binding as a percent of control levels, error bars represent standard errors of means. (A) CI-953 caused complete inhibition of binding at concentrations greater than 300 μM with a K_d of 29 μM and an apparent Hill coefficient of 1.08. (B) Similarly, CI-946 inhibited binding with a K_d of 25 μM and a Hill coefficient of 1.16.

trations. Similar calculations for phenytoin and carbamazepine from the maximal electroshock model with rats[9] agree well with clinical reports that unbound therapeutic concentrations are from 4–8 μM for phenytoin and 3–12.5 μM for carbamazepine[2].

At concentrations of CI-953 or ralitoline well above the 'therapeutic levels' shown in Table I, no effects on electrophysiological responses to GABA, a major inhibitory neurotransmitter, or the excitatory neurotransmitter glutamate, were noted.

The inhibitory action of CI-953 and ralitoline on rapid trains of action potentials from sustained depolarization of cultured spinal cord neurons is similar to results obtained with phenytoin and carbamazepine[11,12] and also similar to results with valproate[13] and zonisamide[19]. All of the drugs that inhibit sustained firing in vitro also prevent tonic extensor seizures from maximal electroshock in rats, and all except valproate have been shown electrophysiologically and by displacement of batrachotoxinin binding to interact with voltage-sensitive sodium channels.

Both phenytoin and carbamazepine in many different voltage clamp preparations have been shown to reduce voltage-dependent inward sodium currents and to shift the voltage dependence of

TABLE II

Effect of ralitoline (CI-946) and CI-953 on sustained repetitive firing in vitro with comparison to unbound plasma levels attained during protection from maximal electroshock seizures in rats

Compound	*IC_{50} sustained firing*	*Unbound plasma level (μM)*
CI-953	5.0	4.3
Ralitoline	2.0	1.1
Phenytoin	4–8[a]	4[b]
Carbamazepine	3–4[a]	5.3[b]

[a] From McLean and Mcdonald, refs. 11,12.
[b] From Masuda et al., ref. 9 and corrections for plasma protein binding of 87% (phenytoin) and 75% (carbamazepine).

TABLE III

Effect of ralitoline (CI-946) and CI-953 on BTX-b binding in rat brain synaptosomes in vitro with comparison to unbound plasma levels attained during protection from maximal electroshock seizures in rats

Compound	*Apparent K_d (μM)*	*Unbound plasma level (μM)*	*Calculated receptor occupancy*
CI-953	29	4.3	0.14
Ralitoline	25	1.1	0.04
Phenytoin	40	4–8[a]	0.22–0.33[b]
Carbamazepine	131	3–12.5[a]	0.02–0.13[b]

[a] From Bruni and Albright, ref. 2.
[b] From Willow and Catterall, ref. 26.

inactivation of sodium currents to more negative voltages[8,10,14,20–22,24,27]. A recent analysis of the action of phenytoin at single sodium channels has shown that phenytoin interacts with both inactivated and resting sodium channels[17]. It has been postulated that these changes in sodium conductance lead to the eventual blockade of sodium action potentials, especially during high frequency firing and sustained depolarizations such as those seen during seizures.

The displacement of BTX-b binding by CI-953 and ralitoline indicates that these drugs modulate binding of neurotoxins to receptor site 2 on sodium channels. This site is highly sensitive to the conformational state of the sodium channel protein and numerous neurotoxins, insecticides, local anesthetics, antiarrhythmic drugs and anticonvulsants alter neurotoxin binding by indirect allosteric interactions[5,26]. Analysis of the competition of binding interactions between carbamazepine and the local anesthetic lidocaine indicates a common site of interaction at the receptor[28]. The binding results extend the association previously made between anticonvulsant activities and allosteric inhibition of BTX-b binding[26].

Table III compares the concentrations of anticonvulsants inhibiting BTX-b binding with unbound plasma concentrations achieved during prevention of seizures in rodents (Bockbrader and VonHodenberg, unpublished observations) or therapeutic concentrations in clinical studies with phenytoin and carbamazepine. Phenytoin, carbamazepine and CI-953 each occupy between 13% and 33% of sodium channel sites at therapeutic concentrations. Ralitoline occupied approximately 4% of sodium channel sites. Other anticonvulsants (valproate, phenobarbital, diazepam) had little effect on BTX-b binding at therapeutic concentrations[26]. The actual occupancy of these lipophilic drugs may be higher, since they may significantly partition into brain membranes and increase concentration at sodium channels. Moreover, since inhibition of sodium channels is strongly frequency and voltage dependent[10,17,21,27], the effective K_d in an epileptic focus may be more potent than in binding experiments with synaptosomes. It will be interesting to examine the frequency and voltage dependence of CI-953 and ralitoline for inhibition of voltage-clamped sodium currents.

In summary, our results show inhibition of sustained repetitive action potentials from neuronal membrane depolarization and also inhibition of BTX-b binding from rat synaptosomal membranes by both CI-953 and ralitoline at concentrations that are relevant for the anticonvulsant actions of these compounds in animals. We conclude that the anticonvulsant mechanism of these two compounds is likely to be very similar to that of phenytoin and carbamazepine, and may involve modulation of neuronal voltage-sensitive sodium channels.

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