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Na^+ channel effects of remacemide and desglycinyl-remacemide in rat cortical synaptosomes

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Abstract

The effects of the novel anticonvulsant, remacemide hydrochloride and its active metabolite, desglycinyl-remacemide, on veratridine-induced Na^+ influx in rat cortical synaptosomes were investigated and compared to established Na^+ channel blocking antiepileptic drugs. Remacemide and desglycinyl-remacemide reduced veratridine-stimulated Na^+ influx to 30.7% (IC_{50} = 160.6 μM) and 13.2% (IC_{50} = 85.1 μM) of control, respectively. Carbamazepine, phenytoin and lamotrigine similarly reduced Na^+ influx to 20.1% (IC_{50} = 325.9 μM), 79.8% and 27.9% (IC_{50} = 23.0 μM) of control, respectively. Resting internal Na^+ concentrations were significantly increased by desglycinyl-remacemide (1 and 10 μM) and, conversely, decreased by desglycinyl-remacemide and carbamazepine (both 1000 μM). These studies support previous electrophysiological investigations, which suggest that remacemide and desglycinyl-remacemide exert their antiepileptic effects, at least in part, by an inhibitory action on voltage-gated Na^+ channels. Desglycinyl-remacemide may have an additional action on Na^+ homeostasis that merits further exploration.

Keywords: Remacemide; Desglycinyl-remacemide; Antiepileptic drug; Na^+ channel; Synaptosome, Rat

1. Introduction

Remacemide hydrochloride is a novel anticonvulsant agent with efficacy in a wide range of experimental seizure models (Clark et al., 1995; Davies, 1997). Remacemide and its active metabolite, desglycinyl-remacemide, demonstrate weak channel blocking activity at the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor (Palmer et al., 1992; Hu and Davies, 1995). Electrophysiological studies in cultured mouse spinal cord neurones (Wamil et al., 1996) and rat hippocampal slices (Norris and King, 1997) suggest that both agents have an additional action on voltage-dependent Na^+ channels.

Blockade of voltage-gated Na^+ channels is a recognised mechanism of action of several commonly used antiepileptic drugs, including carbamazepine, phenytoin and lamotrigine (Rogawski and Porter, 1990; White, 1999; Kwan et al., 2001). A plethora of electrophysiological investigations suggest that these agents produce a characteristic voltage- and use-dependent blockade of Na^+ channels, reducing high-frequency neuronal firing with little or no effect on the amplitude or duration of single action potentials (Willow et al., 1985; Schwartz and Grigat, 1989; Ragsdale et al., 1991; Lang et al., 1993; Lees and Leach, 1993; Kuo and Bean, 1994; Xie et al., 1995; Kuo, 1998). Binding studies (Willow and Catterall, 1982) and numerous neurochemical investigations (Willow et al., 1984; Leach et al., 1986; Waldmeier et al., 1995; Deffois et al., 1996; Lingamaneni and Hemmings, 1999) substantiate these observations.

The Na^+ channel effects of remacemide and desglycinyl-remacemide are, however, considerably less well characterised. There is evidence to support inhibition of sustained repetitive firing of Na^+-dependent action potentials (Wamil et al., 1996; Norris and King, 1997) and an interaction with [^{3}H]batrachotoxin binding (Palmer et al., 1992). However, specific neurochemical investigations of Na^+ channel blockade with remacemide and desglycinyl-remacemide are limited (Srinivasan et al., 1995) and the results potentially confounding (Davies, 1997).

Accordingly, the following study was designed to characterise the Na^+ channel blocking properties of remacemide and desglycinyl-remacemide using a simple neurochemical technique in rat brain synaptosomes. Carbamazepine, phenytoin and lamotrigine were included for comparative purposes.

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2. Materials and methods

2.1. Materials

Remacemide ((±)-2-amino-*N*-(1-methyl-1,2-diphenylethyl) acetamide hydrochloride) and desglycinyl-remacemide ((±)-1-methyl-1,2-diphenylethylamine) were obtained from AstraZeneca R&D Charnwood (Loughborough, UK). Lamotrigine (6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine) was obtained from GlaxoWellcome Research and Development (Stevenage, UK). All other reagents, including carbamazepine (5*H*-dibenz[*b*,*f*]azepine-5-carboxamide) and phenytoin (5,5-diphenyl-2,4-imidazolidinedione), were obtained from Sigma (Poole, UK).

2.2. Preparation of synaptosomes

Synaptosomes were prepared by a modification of the method of Urenjak et al. (1991). All manipulations were performed at 4 °C, unless otherwise stated. Adult male Wistar rats (200–250 g; Harlan Olac, Bicester, UK) were sacrificed by cervical dislocation, the brain removed and cerebral cortex dissected free (Glowinski and Iversen, 1966). This procedure was performed in accordance with the Animals (Scientific Procedures) Act, 1986 (UK). Cortical tissue was homogenised with a motorised teflon pestle in 15 ml homogenisation buffer (0.32 M sucrose, 5 mM Na-2-([2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]-amino)ethanesulphonate, 0.5 mM ethylenediaminetetra-acetic acid (EDTA), 16 μM bovine serum albumin; pH 7.4). The homogenate was centrifuged at 1000 × *g* for 10 min. The resulting supernatant was centrifuged at 22,000 × *g* for 20 min to produce a crude mitochondrial/synaptosomal pellet. The pellet was resuspended in 4 ml of homogenisation buffer, layered on a discontinuous Ficoll gradient (2.5 ml 12% Ficoll; 1.5 ml 9% Ficoll; 2.5 ml 6% Ficoll) and centrifuged at 90,000 × *g* for 60 min.

The two synaptosomal layers identified within the 9% Ficoll region were removed, washed with 18 ml homogenisation buffer (minus EDTA) and centrifuged for 20 min at 55,000 × *g*. The resulting pellet was resuspended in 9.96 ml of standard Na^+-free incubation medium (3 mM KCl, 2 mM $MgCl_2$, 25 mM HEPES, 10 mM glucose and 140 mM sucrose; pH 7.4) and stored on ice.

2.3. Dye loading

Synaptosomes were loaded with Na^+ sensitive dye, Na^+-binding benzofuran isophthalate acetoxymethylester (SBFI-AM), according to the method of Deri and Adam-Vizi (1993). A 20 μl aliquot of 2 mM SBFI-AM was mixed with 20 μl of 2% pluronic F-127 and added to the synaptosomal suspension. The presence of detergent (pluronic F-127) facilitates uptake of SBFI-AM into the synaptosomes. Dye loading was performed in a light-protected vessel at 37 °C, with continuous mixing, for a period of 70 min. During the loading period, SBFI-AM accumulates in the synaptosomes where it is hydrolysed to the Na^+ sensitive form, SBFI. Loading efficiency was monitored in a 2 ml aliquot of the suspension by fluorescence detection. To correct for the potential leakage of dye, a fluorescence intensity ratio was determined between excitation at 340 nm, the Na^+-dependent intensity, and at 380 nm, the Na^+-independent intensity, as reported by Harootunian et al. (1989) and Borin and Siffert (1990).

Following loading, the synaptosomal suspension was centrifuged at 11,000 × *g* for 10 min. Unloaded dye was removed by washing the pellet in standard Na^+-free incubation medium. This suspension was centrifuged for 5 min at 12,000 × *g* and the pellet finally resuspended in standard Na^+-free incubation medium to a protein concentration of 6 mg/ml (Biorad method; Sills et al., 1997).

2.4. Standard curve

A standard curve, relating the 340/380 nm fluorescence intensity ratio to Na^+ concentration, was prepared in the presence of 2 μM gramicidin D, an ionophore that renders plasma membranes permeable to monovalent cations (Keen and White, 1971). In the presence of ionophore, it was assumed that internal and external Na^+ concentrations were equivalent. Synaptosomes were exposed to known Na^+ concentrations (12–200 mM), in the presence of 2 μM gramicidin D, for 5 min (at 37 °C) and the 340/380 nm fluorescence intensity ratio (at an emission of 510 nm) was recorded (Fig. 1).

2.5. Determination of resting Na^+ concentration

To investigate drug effects on resting internal Na^+ concentration, 100 μl of the synaptosomal suspension was added to 1.85 ml of standard incubation medium (37 °C), supplemented with 140 mM NaCl and 2 mM $CaCl_2$, and containing the appropriate drug concentration. The 340/380

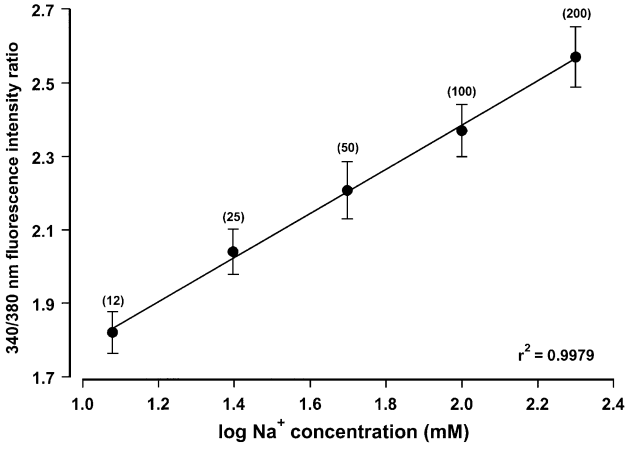

Fig. 1. Relationship between absolute Na^+ concentration (mM; in parentheses) and 340/380 nm fluorescence ratio in rat cortical synaptosomes in the presence of the Na^+ ionophore gramicidin D ($n = 5$). Linearity was determined by simple regression analysis.

nm fluorescence intensity ratio was noted at 0 and 5 min. The internal Na^+ concentration at 5 min was deemed to be the resting concentration for the preparation and served as the control value for subsequent veratridine-induced influx investigations.

2.6. Veratridine-stimulated Na^+ influx

Veratridine activates voltage-dependent Na^+ channels, causing Na^+ influx and membrane depolarisation (Narahashi, 1974). Following determination of resting Na^+ concentration (see above), 50 μl veratridine was added to the fluorescence cell (final concentration = 200 μM). After a 5-min incubation at 37 °C, the 340/380 nm fluorescence intensity ratio was recorded.

2.7. Validation

To confirm assay specificity, a validation was performed using the prototypic Na^+ channel blocker tetrodotoxin (Narahashi, 1974). Synaptosomes were prepared and loaded with dye as described above. The effects of tetrodotoxin (0–10 μM) on resting Na^+ concentrations and veratridine-stimulated Na^+ influx were determined.

2.8. Data analysis

Analyses were performed using Minitab for Windows version 10.1 on a Viglen P2 400 microcomputer. Resting Na^+ results ($n = 5$) were expressed as the mean (± S.E.M.) percentage of control concentration in individual preparations and compared by one-way analysis of variance with Dunnett correction for multiple comparisons. Results from veratridine experiments ($n = 5$) were expressed as the mean (± S.E.M.) percentage of control response in individual preparations. IC_{50} values were determined by log transformation of the data and subsequent linear regression analysis.

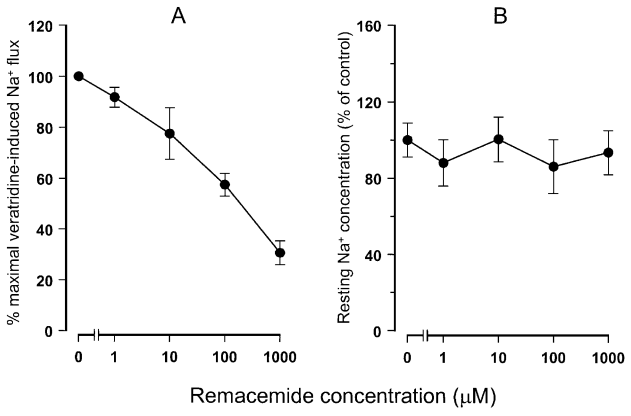

Fig. 3. (A) Effect of remacemide (0–1000 μM) on veratridine-induced Na^+ influx in rat cortical synaptosomes. Results ($n = 5$) are expressed as the mean percentage (± S.E.M.) of the maximal response to veratridine. (B) Effect of remacemide (0–1000 μM) on resting internal Na^+ concentration in rat cortical synaptosomes. Results ($n = 5$) are expressed as the mean percentage (± S.E.M.) of control concentration.

3. Results

3.1. Validation

Tetrodotoxin reduced veratridine-stimulated Na^+ influx to 6.3% (± 5.5) of control ($IC_{50} = 102.2$ nM; Fig. 2).

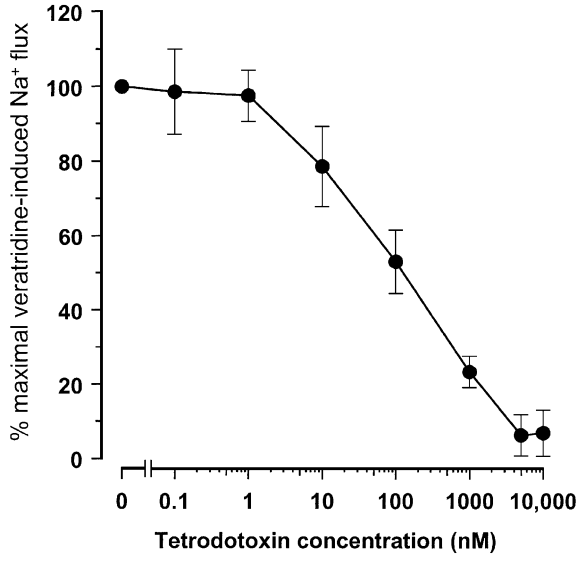

Fig. 2. Effect of tetrodotoxin (0–10 μM) on veratridine-induced Na^+ influx in rat cortical synaptosomes. Results ($n = 5$) are expressed as the mean percentage (± S.E.M.) of the maximal response to veratridine.

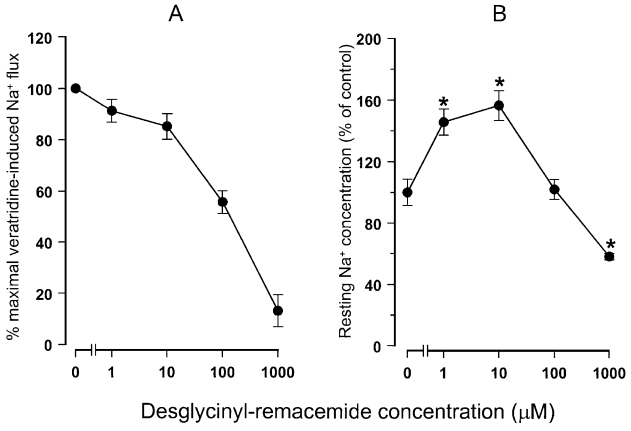

Fig. 4. (A) Effect of desglycinyl-remacemide (0–1000 μM) on veratridine-induced Na^+ influx in rat cortical synaptosomes. Results ($n = 5$) are expressed as the mean percentage (± S.E.M.) of the maximal response to veratridine. (B) Effect of desglycinyl-remacemide (0–1000 μM) on resting internal Na^+ concentration in rat cortical synaptosomes. Results ($n = 5$) are expressed as the mean percentage (± S.E.M.) of control concentration and statistical significance (* $P < 0.05$) was determined by one-way analysis of variance with Dunnett correction for multiple comparisons.

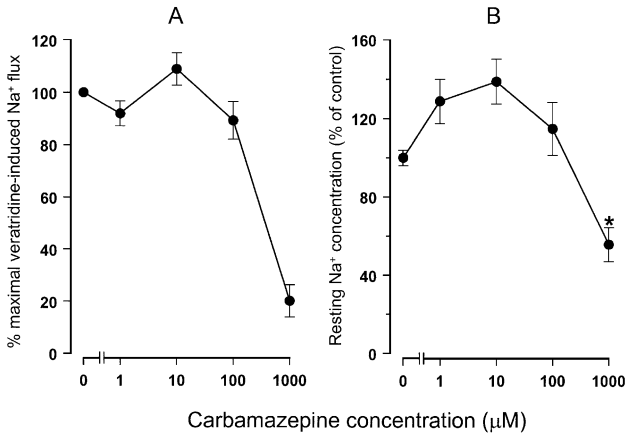

Fig. 5. (A) Effect of carbamazepine (0–1000 μM) on veratridine-induced Na^+ influx in rat cortical synaptosomes. Results ($n=5$) are expressed as the mean percentage (± S.E.M.) of the maximal response to veratridine. (B) Effect of carbamazepine (0–1000 μM) on resting internal Na^+ concentration in rat cortical synaptosomes. Results ($n=5$) are expressed as the mean percentage (± S.E.M.) of control concentration and statistical significance (* $P<0.05$) was determined by one-way analysis of variance with Dunnett correction for multiple comparisons.

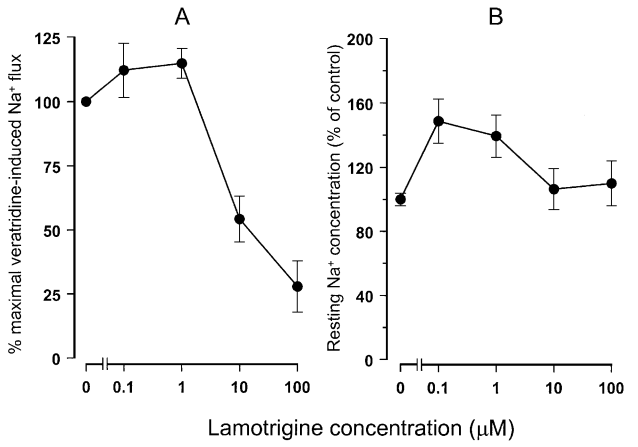

Fig. 7. (A) Effect of lamotrigine (0–100 μM) on veratridine-induced Na^+ influx in rat cortical synaptosomes. Results ($n=5$) are expressed as the mean percentage (± S.E.M.) of the maximal response to veratridine. (B) Effect of lamotrigine (0–100 μM) on resting internal Na^+ concentration in rat cortical synaptosomes. Results ($n=5$) are expressed as the mean percentage (± S.E.M.) of control concentration.

Tetrodotoxin was without effect on resting Na^+ concentrations (data not shown).

3.2. Veratridine-stimulated Na^+ influx

Remacemide (Fig. 3A) and desglycinyl-remacemide (Fig. 4A) reduced veratridine-stimulated Na^+ influx to 30.7% (± 4.7) and 13.2% (± 6.2) of control, with IC_{50} values of 160.6 and 85.1 μM, respectively. Carbamazepine (Fig. 5A), phenytoin (Fig. 6A) and lamotrigine (Fig. 7A) similarly reduced veratridine-stimulated Na^+ influx to 20.1% (± 6.2), 79.8% (± 6.6) and 27.9% (± 10.0) of control. IC_{50} values for carbamazepine and lamotrigine were 325.9 and 23.0 μM, respectively.

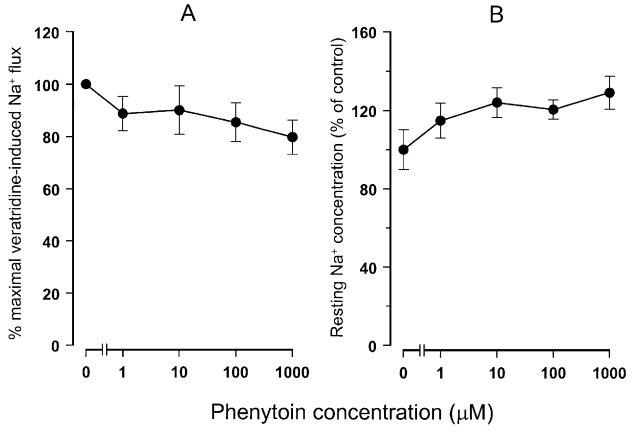

Fig. 6. (A) Effect of phenytoin (0–1000 μM) on veratridine-induced Na^+ influx in rat cortical synaptosomes. Results ($n=5$) are expressed as the mean percentage (± S.E.M.) of the maximal response to veratridine. (B) Effect of phenytoin (0–1000 μM) on resting internal Na^+ concentration in rat cortical synaptosomes. Results ($n=5$) are expressed as the mean percentage (± S.E.M.) of control concentration.

3.3. Resting Na^+ concentrations

The mean resting internal Na^+ concentration was calculated at 22.5 mM (± 2.0; $n=25$). Desglycinyl-remacemide (1 and 10 μM) significantly ($P<0.05$) increased the resting Na^+ concentration (Fig. 4B). In contrast, a significant ($P<0.05$) decrease in resting Na^+ levels was observed with 1000 μM desglycinyl-remacemide (Fig. 4B) and 1000 μM carbamazepine (Fig. 5B). Remacemide (Fig. 3B), phenytoin (Fig. 6B) and lamotrigine (Fig. 7B) were without effect.

4. Discussion

Remacemide is a novel anticonvulsant compound with weak channel blocking activity at the NMDA subtype of glutamate receptor (Palmer et al., 1992; Hu and Davies, 1995). Electrophysiological studies suggest that remacemide, and its active metabolite desglycinyl-remacemide, have additional inhibitory effects on voltage-dependent Na^+ channels (Wamil et al., 1996; Norris and King, 1997), although substantiating neurochemical data is limited (Srinivasan et al., 1995). This investigation was designed to confirm the Na^+ channel effects of remacemide and desglycinyl-remacemide using a simple neurochemical assay in rat brain synaptosomes.

Assay validation with the prototypic Na^+ channel blocker tetrodotoxin confirmed the specificity of the technique and its suitability for the study of voltage-gated Na^+ channels and their pharmacological blockade. Subsequent studies with remacemide and desglycinyl-remacemide suggested that both agents blocked voltage-gated Na^+ channels

in a concentration-dependent manner. The reference compounds, carbamazepine, phenytoin and lamotrigine, similarly reduced veratridine-stimulated Na^+ influx, in accordance with their reported mechanisms of action. In terms of potency, however, a wide variability was observed and the relative inactivity of phenytoin difficult to explain.

Previous studies suggest that both remacemide and desglycinyl-remacemide displace [^{3}H]batrachotoxin binding to voltage-gated Na^+ channels with IC_{50} values of 15.6 and 7.9 μM, respectively (Palmer et al., 1992). In cultured mouse spinal cord neurones, remacemide and desglycinyl-remacemide reduce sustained repetitive firing, an electrophysiological marker for voltage-gated Na^+ channel activity, with IC_{50} values of 7.9 and 1.2 μM, respectively (Wamil et al., 1996). A similar study, recording sustained repetitive firing of CA1 neurones in the intact hippocampal slice preparation, reported half maximal inhibitory concentrations of 66 and 60 μM for remacemide and desglycinyl-remacemide, respectively (Norris and King, 1997). Although these findings largely support the two-fold separation in potency between remacemide and desglycinyl-remacemide in the current investigation, when IC_{50} values are compared, questions of pharmacological sensitivity arise.

However, similarly disparate results are reported from a surfeit of studies with other Na^+ channel blocking antiepileptic drugs. Carbamazepine reduces sustained repetitive firing in cultured mouse central neurones with an IC_{50} of 4 μM (McLean and Macdonald, 1986) and prevents batrachotoxin-induced $^{22}Na^+$ flux into rat brain synaptosomes at 22 μM (Willow et al., 1984). In addition, it displaces [^{3}H]batrachotoxin binding with an IC_{50} of 131 μM (Willow and Catterall, 1982) and prevents veratridine-induced glutamate release from rat brain synaptosomes at 200 μM (Lingamaneni and Hemmings, 1999). A similar range of potencies have been reported from multiple and diverse studies with phenytoin (Willow and Catterall, 1982; Willow et al., 1984, 1985; Lang et al., 1993; Deffois et al., 1996; Lingamaneni and Hemmings, 1999) and lamotrigine (Leach et al., 1986; Lang et al., 1993; Lees and Leach, 1993; Waldmeier et al., 1995; Xie et al., 1995; Lingamaneni and Hemmings, 1999).

In order to understand wide variations in potency when comparing the findings of pharmacological studies of Na^+ channel blockade, it is important to appreciate that Na^+ channels are considerably more sensitive to antiepileptic drug-mediated amelioration under depolarising conditions (Rogawski and Porter, 1990). This reflects the significantly greater affinity of these agents for the inactivated state of the channel and is fundamental in their ability to reduce high-frequency neuronal firing with little or no effect on the amplitude or duration of single action potentials (Kuo, 1998). As such, electrophysiological investigations of the voltage- and use-dependent blockade of Na^+ channels by antiepileptic drugs are consistently more sensitive than neurochemical studies that are usually conducted under physiological conditions and accordingly restricted to reporting tonic inhibitory effects of antiepileptic drugs on Na^+ channel function.

The results of this study, and countless previous investigations, suggest that, at therapeutic concentrations, the Na^+ channel effects of antiepileptic drugs are restricted to voltage- and use-dependent blockade. Tonic inhibition of Na^+ channels, with the potential to influence action potential generation, is only observed at supra-therapeutic concentrations. There is no evidence to suggest that antiepileptic drugs influence Na^+ homeostasis at the resting membrane potential. In this study, however, desglycinyl-remacemide (1 and 10 μM) significantly increased resting internal Na^+ concentration. A contrasting decrease was observed with both desglycinyl-remacemide and carbamazepine (1000 μM), although this finding can be discounted on the basis of supra-therapeutic drug toxicity. The effect of desglycinyl-remacemide on resting internal Na^+ concentration is unlikely to be related to voltage-gated Na^+ channel blockade, given the wide separation in effective concentrations. It is, however, possible that desglycinyl-remacemide has a weak pharmacological action on one of the many transport systems, such as Na^+/K^+ ATP-ase and the Na^+/H^+ antiport, which maintain the Na^+ ion gradient across the cell membrane. This hypothesis requires further detailed investigation.

In conclusion, the novel anticonvulsant compound remacemide and its active metabolite desglycinyl-remacemide reduce voltage-gated Na^+ channel activity in a concentration-related manner. Previous electrophysiological investigations have reported voltage- and use-dependent blockade of Na^+ channels with these agents (Wamil et al., 1996; Norris and King, 1997). This neurochemical study supports these findings and suggests that tonic inhibition of Na^+ channel function is only observed at supra-therapeutic concentrations. Given the relative potencies and steady-state concentrations of both agents following remacemide administration in man, it is likely that the desglycinyl metabolite is the principal pharmacological moiety and NMDA receptor blockade the primary mechanism of action (Davies, 1997). Nevertheless, inhibition of voltage-gated Na^+ channels by remacemide undoubtedly contributes to its anticonvulsant effect.

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