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# Effects of besipirdine at the voltage-dependent sodium channel

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1 Besipirdine (HP 749) is a compound undergoing clinical trials for efficacy in treating Alzheimer's disease. Among other pharmacological effects, besipirdine inhibits voltage-dependent sodium and potassium channels. This paper presents a pharmacological study of the interaction of besipirdine with voltage-dependent sodium channels.

2 Besipirdine inhibited [$^3$H]-batrachotoxin binding ($IC_{50} = 5.5 \pm 0.2$ $\mu$M) in a rat brain vesicular preparation and concentration-dependently inhibited veratridine (25 $\mu$M)-stimulated increases in intracellular free sodium ($[Na^+]_i$) and calcium ($[Ca^{2+}]_i$) in primary cultured cortical neurones of rat.

3 Besipirdine (30-100 $\mu$M) concentration-dependently inhibited (up to 100%) veratridine-stimulated release of [$^3$H]-noradrenaline (NA) from rat cortical slices.

4 When examined in greater detail, besipirdine was found to inhibit [$^3$H]-batrachotoxin binding in vesicular membranes competitively. However, when examined in rat brain synaptosomes, we found that the antagonism by besipirdine was not competitive; that is, the maximal stimulation of $[Ca^{2+}]_i$ induced by veratridine decreased with increasing concentrations of besipirdine.

5 These results show that besipirdine is an inhibitor of voltage-sensitive sodium channels and appears to bind to a site close to the batrachotoxin/veratridine binding site.

**Keywords:** Besipirdine; sodium channel; anticonvulsant; veratridine; batrachotoxin; local anaesthetic; intracellular sodium; intracellular calcium; neurotransmitter release

## Introduction

Besipirdine HCl (HP 749; N-propyl-N-(4-pyridinyl)-1H-indol-1-amine hydrochloride) is a compound currently undergoing clinical trials for efficacy in treating Alzheimer's disease, a progressive neurological disorder affecting an increasing number of people worldwide (Evans *et al.*, 1989). It is characterized by a gradual deterioration of cognitive function and deficits in multiple neurotransmitter systems. Besipirdine reverses memory deficits caused by disruption of cholinergic and noradrenergic systems in animal behavioural models (Santucci *et al.*, 1991). Reports regarding the *in vitro* and *in vivo* pharmacological properties of besipirdine have described effects on multiple neurotransmitter systems (Cornfeldt *et al.*, 1990; Huger *et al.*, 1990; Zaczek *et al.*, 1993; Smith *et al.*, 1994a), particularly the cholinergic and noradrenergic systems. Interaction with the voltage-dependent sodium channel was suggested by Smith *et al.* (1994a) in that veratridine-induced, calcium-independent [$^3$H]-noradrenaline ([$^3$H]-NA) release was inhibited by besipirdine (100 $\mu$M) but not by tetrodotoxin (5 $\mu$M). Moreover, besipirdine, at concentrations higher than 10 $\mu$M, has been shown to inhibit inward and outward currents under whole-cell patch clamp conditions, and the pharmacological and electrophysiological properties of besipirdine and the potassium channel inhibitor, 4-animopyridine have been compared (Huger *et al.*, unpublished observations). The results we present here provide a more detailed pharmacological examination of the interaction of besipirdine with voltage-dependent sodium channels.

Voltage-dependent sodium channels have a variety of active sites that have been characterized pharmacologically (Catterall 1977; 1987; Catterall *et al.*, 1981; Brown, 1986; 1988). The alkaloid toxins, veratridine and batrachotoxin bind to a modulatory site (so-called site II) on the sodium channel such that they produce persistent opening of the channel and subsequent depolarization of the cell (Catterall, 1977). Tetrodotoxin and saxitoxin bind to a site close to the channel port and block ion flux through the channel (Catterall, 1986; Brown, 1986), Thus, when bound, tetrodotoxin and saxitoxin block the effects of veratridine and batrachotoxin.

Other known modulators of sodium channels include anticonvulsants such as phenytoin and local anaesthetics such as lignocaine (Catterall, 1987). These compounds inhibit sodium currents and [$^3$H]-batrachotoxin binding (Brown, 1986; Catterall, 1987; Creveling *et al.*, 1983; Sheldon *et al.*, 1994). Despite extensive studies by several groups, the precise site of action of these compounds remains unclear. Some studies report the interaction between these compounds and batrachotoxin to be competitive (Creveling *et al.*, 1983) while others report a mixed competitive/non-competitive profile (Catterall, 1987; Sheldon *et al.*, 1994). When these compounds are examined against a partial agonist such as veratridine, they have been reported to show a non-competitive profile (Catterall *et al.*, 1981).

The experiments described in this paper examined the ability of besipirdine to inhibit [$^3$H]-batrachotoxin binding, and to reverse the effects of veratridine on $[Na^+]_i$, $[Ca^{2+}]_i$ and [$^3$H]-NA release through voltage-dependent sodium channels.

## Methods

### *[$^3$H]-batrachotoxin binding*

Inhibition of [$^3$H]-batrachotoxin was performed by a modification of the procedure of Creveling *et al.* (1983). Briefly, the brain was removed from male Wistar rats and the cortex was separated and placed in a glass homogenizer filled with 12 ml ice-cold HEPES-buffered salt solution (HBS; composition in mM: NaCl 124, KCl 5, $CaCl_2$, 1, $NaHCO_3$ 25.9, $KH_2PO_4$ 1.2, HEPES 10, glucose 10, pH to 7.4 with NaOH). The tissue was homogenized at 3,500 r.p.m. for 6 up and down strokes, and centrifuged at 1,000 *g* for 15 min. The supernatant was discarded and the pellet containing vesicular elements was resuspended in binding medium (composition in mM: choline chloride 130, glucose 5.5, $MgSO_4$ 0.8, KCl 5.4 and HEPES 50,

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pH 7.4). Incubations were carried out in a total volume of 250 μl containing 1 μM tetrodotoxin (Sigma), 20 μg of scorpion venom (Sigma), 25 nM [$^3$H]-batrachotoxin A 20-α benzoate (NEN; Wilmington, DE, U.S.A.), and approximately 400 μg of protein of the vesicular preparation. High affinity [$^3$H]-batrachotoxin binding is dependent on the positive cooperative effect of scorpion venom (Catterall *et al.*, 1981), and tetrodotoxin increases scorpion venom binding by stabilizing the membrane potential. After a 60 min incubation at 25°C, the mixture was diluted with 3 ml of wash medium (composition in mM: choline chloride, 163, $CaCl_2$ 1.8, $MgSO_4$ 0.8 and HEPES 5; pH 7.4) and bound [$^3$H]-batrachotoxin was collected on glass-fibre filters under vacuum. The filters were then washed twice with 3 ml of wash medium and placed in scintillation vials with 5 ml of Ecoscint (National Diagnostics, Atlanta, GA, U.S.A.). The tritium content was measured by scintillation spectroscopy. Veratridine (300 μM; Sigma) was included in some tubes to determine non-specific binding, which was 4.75±0.15% (mean±s.e.) of total binding. Data were analysed by one site non-linear regression fit (Prism, GraphPad, San Diego, CA, U.S.A.).

For saturation binding studies, 1–100 nM of [$^3$H]-batrachotoxin was used in the absence or in the presence of 1 and 5 μM besipirdine. Besipirdine was synthesized at the Department of Chemical Research, Hoechst-Roussel Pharmaceuticals, Inc. as described by Effland *et al.* (1990). Data were analysed by one-site linear regression fit (Scatchard) using Prism.

### *Culture of rat cortical neurones*

Mixed cortical cell cultures containing both neuronal and glial elements were prepared from foetal (18 day old) Sprague Dawley rats as previously described (Choi, 1987). Briefly, dissociated cortical cells were plated in Eagle's minimum essential medium supplemented with 10% horse serum, 2 mM glutamine and 33 mM glucose and plated in 35 mm glass bottom culture dishes previously treated with poly-D-lysine (MatTek, Ashland, MA, U.S.A.). Cultures were maintained at 37°C in an incubator containing 5% $CO_2$ for 5–12 days before being used for fluorescence recording.

### *Synaptosome preparation*

Whole brain (minus cerebellum) synaptosomes were prepared from male Wistar rats by a Percoll gradient method described by Kongsamut & Nachshen (1988). Following the Percoll gradient centrifugation, synaptosomes were resuspended in HBS (one brain into 2 ml) and maintained at 4°C until used.

### *Sodium measurement*

$[Na^+]_i$ was determined using the indicator dye SBFI (sodium-binding benzufuran isophthalate, Molecular Probes, Eugene, OR). Cells were washed once with Krebs buffer then loaded with SBFI by incubation with the acetoxymethyl (AM) ester (5 μM with 0.1% Pluronic F-127) in HBS for 60 min at 37°C. At the end of the loading period, cells were washed twice with HBS then placed in a setup for superfusion on an inverted microscope at room temperature (~22°C). Cells were provided with excitation light alternating between 340 and 380 nm (3 nm slit widths) and emission light was collected through a wide band emission filter (510 nm; Omega Optical, Brattleboro, VT) and measured with a photon counting photomultiplier tube. The 340/380 ratio was calculated and taken as an index of $[Na^+]_i$.

### *Calcium measurements*

Rat cortical neurones were washed once in HBS to remove serum and were incubated with fura2/AM (2 μM; Molecular Probes) for 60 min at 37°C to load them with fura2. At the end of the loading period, cells were washed once with HBS then placed in the microscope setup (at room temperature) for measurement of $[Ca^{2+}]_i$. Excitation light was provided at 340 and 380 nm and emission was measured at 510 nm, as described above. The 340/380 ratio was calculated and taken as an index of $[Ca^{2+}]_i$.

Synaptosomes were loaded with 10 μM fura2/AM for 60 min at 30°C, then centrifuged to wash out excess fura2/AM. After a 15 min period to allow the fura2/AM taken up to be hydrolyzed, experiments were begun. Aliquots (100 μl) of synaptosomes were spun down in a microcentrifuge, resuspended and placed into 2 ml of HBS in a cuvette in a spectrofluorometer (PTI, South Brunswick, NJ, U.S.A.) at room temperature. Fluorescence was monitored with excitation wavelengths alternating between 340 nm and 380 nm and emission set at 510 nm. A 30 s baseline was collected then besipirdine was added; 30 s later, veratridine was added. The ratio of the signals at 340 and 380 nm were calculated and normalized to % inhibition (Figure 3a) or % maximal effect (Figure 3b).

The data points were fitted to the computer generated curves by non-linear least squares regression analysis (Figure 3a,b; Graphpad, Prism). A double reciprocal plot was generated with the data from Figure 3b and fit by linear regression (Figure 3c; Graphpad, Prism).

### *Measurement of neurotransmitter release*

[$^3$H]-NA (35 Ci mmol$^{-1}$, DuPont-NEN) release was measured by a previously described method (Smith *et al.*, 1994a). Briefly, cortical slices (0.4 mm) were pre-incubated in Krebs buffer (composition in mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $KH_2PO_4$ 2.2, $NaHCO_3$ 24.9, $CaCl_2$ 1.3, dextrose 11.1, saturated with 95%$O_2$/5%$CO_2$, pH 7.4) for 30 min at 35°C and then incubated in fresh Krebs buffer containing 25 nM [$^3$H]-NA for 30 min at 35°C. The slices were then placed in glass superfusion chambers (Zahniser *et al.*, 1986) and perfused at 0.75 ml min$^{-1}$ at 37°C; fractions were collected at 7 min intervals. Besipirdine (10–100 μM) was introduced at fraction 5. Veratridine (25 μM) was added at fraction 10. The fractions collected were counted in 10 ml of Liquiscint (National Diagnostics, Atlanta, GA) scintillation fluid and corrected for quench. Tissue slices were dissolved overnight in 0.5 ml of Protosol (DuPont-NEN), buffered with 1 ml of TRIS HCl (Sigma), and counted. Percent fractional release (%FR) was defined as the ratio of tritium released versus the amount present in the tissue.

## Results

Besipirdine inhibited [$^3$H]-batrachotoxin binding with an $IC_{50}$ of 5.53±0.19 μM ($K_i$=3.77 μM; $n$=3). Figure 1 shows a representative Scatchard plot from a saturation binding experiment with and without besipirdine (1 and 5 μM). All lines intersect the x-axis at the same point ($B_{max}$=1.50±0.03 pmol mg$^{-1}$ protein; $n$=3), while the $K_D$ for [$^3$H]-batrachotoxin increases with increasing concentration of besipirdine ($K_D$ in nM: 44.3±1.4 (no besipirdine), 60.4±1.6 (1 μM besipirdine) and 123.1±2.8 (5 μM besipirdine) ($n$= 3)).

Like batrachotoxin, the alkaloid toxin, veratridine, causes persistent opening of sodium channels (Catterall, 1977). Veratridine (25 μM) increased intracellular sodium concentration (Figure 2a) in primary cultured rat cortical neurones immediately after its addition. This increase reached maximum within 30 s and slowly decreased over the next few minutes. When pre-perfused into the culture dishes, besipirdine inhibited the veratridine response in a concentration-dependent fashion. Besipirdine at a concentration of 10 μM inhibited 61.0±7.7% ($n$=3) of the maximum response, whereas a concentration of 100 μM totally blocked the increase in $[Na^+]_i$ induced by veratridine.

The sodium channel-mediated depolarization produced by veratridine also opens voltage-dependent calcium channels leading to a measurable increase in $[Ca^{2+}]_i$. As shown in Figure 2b, veratridine (25 μM) stimulation caused a rapid increase in

$[Ca^{2+}]_i$ of primary cultured rat cortical neurones, and this increase could be inhibited by besipirdine in a concentration-dependent manner (10 $\mu$M besipirdine inhibited 70.5 $\pm$ 6.8% of the maximal response, $n = 3$).

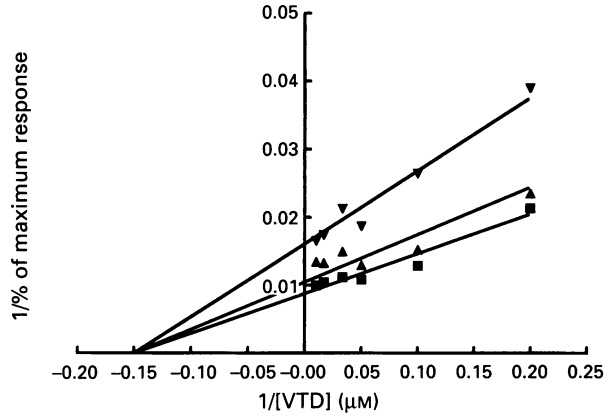


**Figure 1** Besipirdine reduces the $K_D$ for [$^3$H]-batrachotoxin binding without affecting the $B_{max}$. Rat cortical vesicular membranes were incubated with varying concentrations (1 – 100 nM) of [$^3$H]-batrachotoxin in the absence or presence of besipirdine (1 and 5 $\mu$M). Non-specific binding was defined with 500 $\mu$M veratridine. (■) Binding with no besipirdine present; (▲) 1 $\mu$M besipirdine; (▼) 5 $\mu$M besipirdine. Data were analysed by one site linear regression (Scatchard).

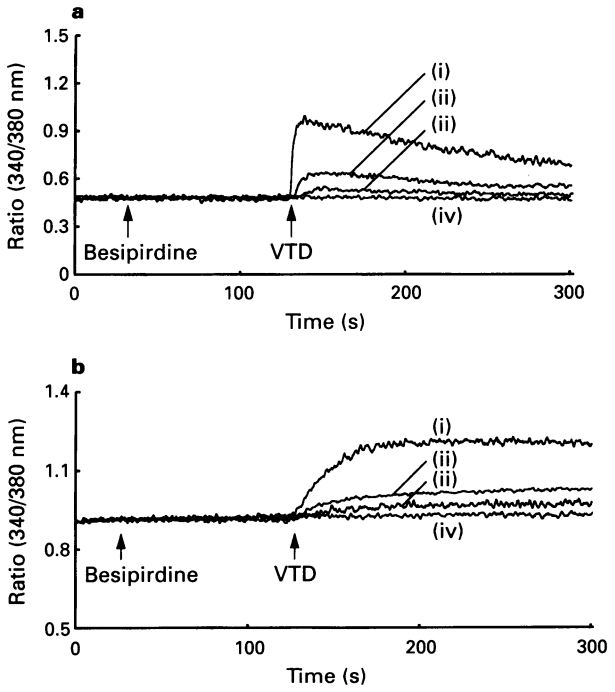


**Figure 2** Besipirdine inhibited veratridine (VTD)-stimulated increases in $[Na^+]_i$ (a) and $[Ca^{2+}]_i$ (b) in primary cultured cortical neurones of rat maintained as described in Methods. The cells were loaded with 5 $\mu$M SBFI/AM (a) or 2 $\mu$M fura2/AM (b) for 60 min at 37°C and then washed once with HBS. Fluorescence was monitored with excitation wavelengths alternating between 340 nm and 380 nm and emission set at 510 nm. Cells were superfused with HBS for 30 s, then the superfusion solution was switched to HBS containing besipirdine (various concentrations); 90 s later, the superfusion solution was again switched to HBS containing veratridine (25 $\mu$M) and besipirdine. The ratios of the signals at 340 and 380 nm were calculated and plotted. Data presented here are a representative example of at least three experiments. (i) Veratridine alone; (ii) 10 $\mu$M besipirdine; (iii) 30 $\mu$M besipirdine; (iv) 100 $\mu$M besipirdine.

We examined the besipirdine-veratridine interaction more carefully in synaptosome preparations. We chose this preparation because a detailed dose-response study requires measurements in a population rather than in single cells and we measured $[Ca^{2+}]_i$ rather than $[Na^+]_i$ because we were unable to obtain adequate loading with SBFI in synaptosomes. Veratridine stimulated increases in $[Ca^{2+}]_i$ in a concentration-dependent manner (Figure 3b). Besipirdine inhibited veratridine (10 and 25 $\mu$M)-stimulated increases in $[Ca^{2+}]_i$ in a concentration-dependent manner (Figure 3a). The $IC_{50}$ values for inhibition by besipirdine were 7.6 $\mu$M and 11.3 $\mu$M for 10 and 25 $\mu$M veratridine, respectively.

Veratridine dose-response curves at two besipirdine concentrations (5 and 10 $\mu$M; Figure 3b) showed a concentration-dependent shift to the right and a decrease in the maximal response to veratridine suggesting that the inhibition may be non-competitive. This was also seen with a double reciprocal plot (Figure 3c; Lehninger, 1982). From this analysis, the $K_A$ for veratridine was 6.67 $\mu$M.

When the interaction of besipirdine and veratridine was examined in measurements of neurotransmitter release, we found that besipirdine inhibited veratridine-induced release of [$^3$H]-NA from rat cortical slices in a concentration-dependent manner. Veratridine (25 $\mu$M) alone increased [$^3$H]-NA release 8 to 10 fold, presumably due to membrane depolarization and extracellular calcium entry (Figure 4). Pretreatment with 10 $\mu$M besipirdine had no measurable effect on the veratridine-induced release. However, 30 $\mu$M besipirdine inhibited 83% and 100 $\mu$M besipirdine completely inhibited the veratridine-induced release. Interestingly, 100 $\mu$M besipirdine alone caused a spontaneous release of [$^3$H]-NA as has been reported previously (Smith *et al.*, 1994a).

## Discussion

The evidence presented in this manuscript demonstrates that besipirdine interacts with voltage-dependent sodium channels. We found that besipirdine displaced the binding of [$^3$H]-batrachotoxin in a rat brain vesicular preparation. Given that batrachotoxin and veratridine open sodium channels, we asked whether besipirdine also opens sodium channels or inhibits the effects of veratridine. We measured $[Na^+]_i$ and $[Ca^{2+}]_i$, and neurotransmitter release and found that besipirdine alone (5 – 30 $\mu$M) had no effect on any of these parameters indicating that it did not open sodium channels. However, it blocked the effects of veratridine in a concentration-dependent fashion suggesting that it may be an antagonist at this site. The concentration dependence of this interaction showed a good correlation across all the measurements made. More detailed competition studies with [$^3$H]-batrachotoxin binding indicated that the interaction between besipirdine and batrachotoxin was competitive; however, studies in rat brain synaptosomes demonstrated that the interaction with veratridine is either non-competitive or indicative of irreversible antagonism. We currently have no data available to distinguish between these two possibilities.

In single primary cultured cortical neurones of rat, besipirdine inhibited the veratridine-stimulated increases in $[Na^+]_i$ and $[Ca^{2+}]_i$ in a concentration-dependent manner (Figure 2a,b). Besipirdine, at 10 $\mu$M, inhibited more than 50% of the maximal response, which correlates well with the $IC_{50}$ value (5.53 $\pm$ 0.19 $\mu$M) obtained in [$^3$H]-batrachotoxin binding experiments. As shown in Figure 2, the concentration-dependence for both $[Na^+]_i$ and $[Ca^{2+}]_i$ measurements was similar. However, the time course of the $[Na^+]_i$ response was more rapid than that for the $[Ca^{2+}]_i$ response, consistent with the idea that veratridine depolarizes the cell leading to opening of voltage-dependent calcium channels and an increase in $[Ca^{2+}]_i$.

Increases in $[Ca^{2+}]_i$ stimulate neurotransmitter release and we measured the release of [$^3$H]-NA from rat cortical slices. Again, besipirdine inhibited veratridine-stimulated [$^3$H]-NA release in a concentration-dependent manner. The dose-re-

sponse relation appears to be shifted slightly towards higher concentrations when release data are compared with $[Na^+]_i$ or $[Ca^{2+}]_i$ data. This approximately 3 fold shift may be explained by the more limited ability of besipridine to penetrate into slices versus cells. Besipridine at 100 μM increased basal [$^3$H]-NA release as has been previously reported (Smith *et al.*, 1994a), and it totally blocked the effect of veratridine. It can be argued that besipridine may also inhibit voltage-dependent calcium channels. This is not likely since besipridine (up to 100 μM) does not inhibit electrically stimulated ($Ca^{2+}$-dependent) [$^3$H]-NA release (Smith *et al.*, 1994a), or $K^+$-stimulated release (Huger *et al.*, unpublished). In addition, besipridine does not displace [$^3$H]-nitrendipine binding (Huger *et al.*, 1990), indicating that it does not bind to this site on the L-type calcium channel.

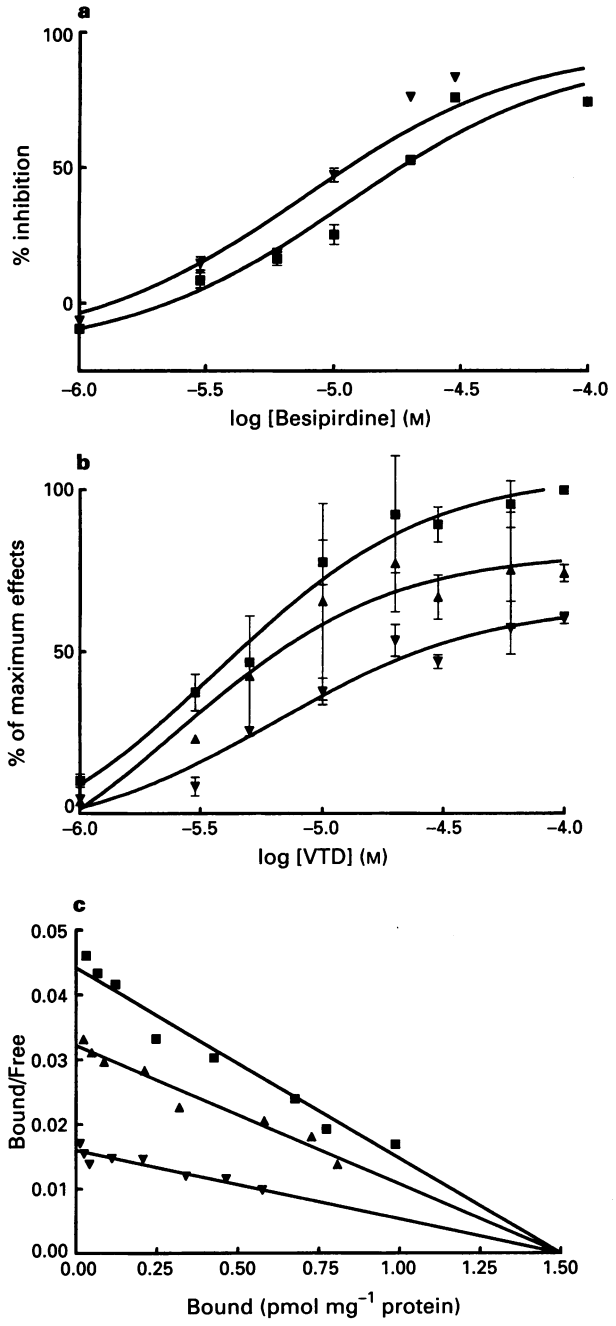


**Figure 3** Besipridine inhibited veratridine-stimulated increases in $[Ca^{2+}]_i$ from rat brain synaptosomes in a concentration-dependent manner and this interaction is non-competitive. Rat brain synaptosomes were prepared as described in Methods. They were then loaded with 10 μM fura2/AM for 60 min at 30°C. Aliquots (100 μl) of synaptosomes were washed once and placed into 2 ml of Krebs buffer in a cuvette for fluorescence measurements. A 30 s baseline was collected before the besipirdine was added; 90 s later, veratridine was added. The ratios of the signals at 340 and 380 nm were calculated and analysed. Data are means ± s.e. ($n=3$). (a) Besipirdine inhibited veratridine-stimulated $[Ca^{2+}]_i$ in a concentration-dependent fashion: (▼) 10 μM and (■) 25 μM veratridine (VTD). (b) With increasing concentrations of besipirdine, the maximal effect of veratridine was decreased. Data are presented as a % of the maximal effect of veratridine at 100 μM. (■) Control, no besipirdine present; (▲), 5 μM besipirdine; (▼) 10 μM besipirdine. (c) A double reciprocal linear plot of data from (b) shows a typical non-competitive profile. $K_A$ for veratridine is 6.67 μM. (■) Control, no besipirdine present; (▲) 5 μM besipirdine; (▼) 10 μM besipirdine.

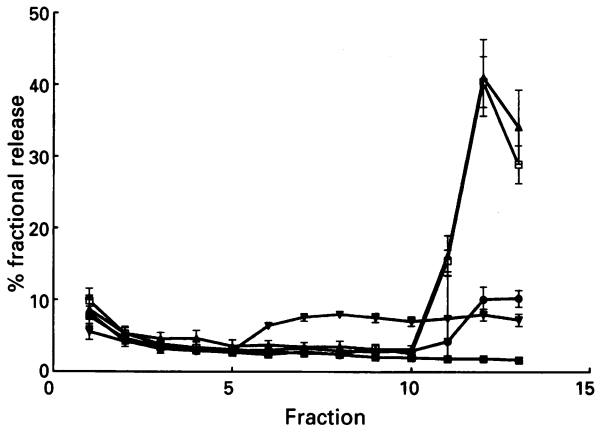


**Figure 4** Besipirdine inhibited veratridine-induced [$^3$H]-NA release from rat cortical slices. Rat cortical slices were perfused as described in Methods. Besipirdine (10–100 μM) was introduced at fraction 5, and veratridine (25 μM) was added at fraction 10. (■) Control, no veratridine stimulation; (□) 25 μM veratridine alone; (▲) 10 μM besipirdine with 25 μM veratridine; (●) 30 μM besipirdine with 25 μM veratridine; (▼) 100 μM besipirdine with 25 μM veratridine. Data presented here are means ± s.e. ($n=3$).

Besipirdine is a structural analogue of 4-aminopyridine and, like 4-aminopyridine, besipirdine inhibits outward currents (Huger *et al.*, unpublished). Besipirdine in addition inhibits inward currents and this inhibition was found to be voltage-dependent (Huger *et al.*, unpublished). The effects on outward and inward current occurred in the same concentration range (10–100 μM).

Besipirdine is currently undergoing clinical trials for efficacy in treating Alzheimer's disease. It has been shown to reverse memory deficits caused by disruption of cholinergic and noradrenergic systems in animal models (Santucci *et al.*, 1991). Any possible link between the interaction with sodium channels described in this manuscript and memory deficit reversal remains to be determined.

Modulators of voltage-dependent sodium channels have already been used as therapeutic agents for other diseases. Anticonvulsants, such as dilantin and carbamazepine, and local anaesthetics, such as lignocaine, are well characterized examples (Catterall, 1987). Other compounds such as flunarizine and its analogues (Roufos *et al.*, 1994), and lamotrigine (Lang *et al.*, 1993) also have effects on sodium channels. Like besipirdine, they displace [$^3$H]-batrachotoxin binding with relatively high $IC_{50}$s, and inhibit sodium currents (Fischer *et al.*, 1992; Lang *et al.*, 1993; Roufos *et al.*, 1994; Sheldon *et al.*, 1994; Huger *et al.*, unpublished). Besipirdine inhibited electroshock-induced seizure in mice (Woodbury & Davenport, 1952) with an $ED_{50}$ of 14.2 ($n=3$) mg kg$^{-1}$ (Szewczak & Huger, personal communication). The structure of besipirdine is unlike that of known anticonvulsants and local anaesthetics. Thus, it is a new modulator of voltage-dependent sodium channels.

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