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Vincamine and vincanol are potent blockers of voltage-gated Na^+ channels

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Abstract

The effects of three vinca derivatives on [^{3}H]batrachotoxin binding in rat cortical synaptosomes, on the inhibition of whole-cell Na^+ currents evoked in voltage-clamped cortical neurones of the rat, on the protection against veratridine-induced cell death in cortical cultures and on the maximal electroshock-induced seizures in mice were compared. Vinpocetine, vincamine and vincanol reduced [^{3}H]batrachotoxin binding with IC_{50} values of 0.34, 1.9 and 10.7 μM, blocked Na^+ currents with IC_{50} values of 44, 72 and 40 μM, and protected cortical cultures against veratridine-induced cell death with IC_{50} values of 0.49, 26 and 33 μM, respectively. Upon i.p. administration, vinpocetine, vincamine and vincanol attenuated maximal electric shock-induced convulsions in a dose-dependent manner with ED_{50} values of 27, 15.4 and 14.6 mg/kg, respectively. The present findings indicate that the three vinca derivatives are potent blockers of voltage-gated Na^+ channels, a mechanism that may contribute at least in part to the pharmacological/therapeutic benefit of these drugs.

Keywords: Vincamine; Vincanol; Vinpocetine; Na^+ channel; Voltage-gated; Batrachotoxin; Neuroprotection; Anticonvulsant

1. Introduction

A growing body of evidence suggests that inhibitors of voltage-gated Na^+ channels are potent neuroprotective agents (Taylor and Meldrum, 1995). Recent studies from this laboratory indicate that the vinca derivative, vinpocetine, is a specific blocker of voltage-gated Na^+ channels (Molnár and Erdõ, 1995) that may efficiently protect cultured cortical neurones against veratridine-induced cell death (Lakics et al., 1995a). This mechanism has been proposed to contribute to the well-documented neuroprotective and/or nootropic efficacy of the drug.

Vinca derivatives of pharmacological relevance can be classified into two major groups: (1) cytotoxic alkaloids of *Vinca rosea*, e.g., vinblastine and vincristine, utilised as antitumour agents; and (2) vincamine, the major alkaloid of *Vinca minor*, and its derivatives, vincanol and vinpocetine possessing (cerebral) vasodilator/nootropic activity (for a review, see Szporny, 1977). Vincamine and vincanol also exhibit neuroprotective properties, like that of vinpocetine (Blasteri et al., 1987). Thus, it was reasonable to assume that vincamine and vincanol may also inhibit voltage-gated Na^+ channels. The present study was an attempt to compare, using in vitro and in vivo methods, the effects of the three vinca derivatives on Na^+ channel-related events.

2. Materials and methods

2.1. Chemicals

Vinpocetine, vincamine and vincanol were kindly provided by Gedeon Richter (Budapest, Hungary). Drug solutions were freshly prepared on the day of each experiment. Drugs were solubilized with a stoichiometric amount of ascorbic acid, or with a drop of 0.1 M HCl and diluted in distilled water, then the pH value of the stock solution was adjusted with NaOH to 6–6.5. [^{3}H]Batrachotoxin (34 Ci/mmol) was purchased from New England Nuclear. All

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other chemicals were of commercial origin and of analytical grade.

2.2. [^{3}H]Batrachotoxin-binding assay

Synaptosomes were prepared from whole brain (except cerebellum) of adult male SPRD rats using discontinuous sucrose gradient centrifugation according to the method of Postma and Catterall (1984). In brief, synaptosomes (200 μg protein/150 μl volume) were incubated for 30 min at 36°C with 13 nM [^{3}H]batrachotoxin in an assay buffer of the following composition: 130 mM choline chloride, 5.5 mM glucose, 0.8 mM $MgSO_4$, 5.4 mM KCl, 1 mg/ml bovine serum albumin 150 mM Hepes-Tris (pH 7.4) in the presence of 1 μM tetrodotoxin, 20 μg/ml scorpion venom (*Leiurus quinquestriatus*) and various concentrations of test drugs. Incubation was terminated by rapid filtration using a Skatron cell harvester. Non-specific binding was determined in the presence of 400 μM aconitine. Protein content was measured by Bio-Rad assay.

2.3. Primary cortical cultures

Primary cultures were prepared from embryonic rat cerebral cortex using a method described elsewhere (Erdõ et al., 1990; Lakics et al., 1995a). Briefly, cells were isolated from 17-day-old rat (Sprague-Dawley, Charles River) foetuses, plated onto poly-D-lysine-coated glass coverslips placed into 24-well plates at a density of 3×10^5 cells per well, in a volume of 0.5 ml. Cultures were maintained for up to 14 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, in a humidified 5% CO_2 atmosphere.

2.4. Patch-clamp experiments

Voltage-clamp recordings were performed at room temperature on the stage of an inverted phase-contrast microscope (Olympus, IMT2). Coverslips with the cultured cells (7–14 days in culture) were transferred into the recording chamber, rinsed and continuously perfused by gravity with an extracellular solution containing (in mM) NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, Hepes 10, glucose 20, sacharose 10 (pH 7.34). Patch pipettes (5–10 MΩ) were prepared from filament containing standard-wall borosilicate glass capillaries (o.d. = 1.2 mm, Clark Electromedicals) with a micropipette puller (P-87, Sutter). The intracellular solution contained (in mM) CsF 130, NaCl 15, tetraethylammonium-Cl 10, $CaCl_2$ 0.1, $MgCl_2$ 2, ATP 2, Hepes 10, EGTA 1 (pH 7.25).

Whole-cell currents were recorded with an Axopatch 200A amplifier using the pClamp 5.5 software (Axon). Signals were filtered at 5 kHz and sampled at 20 kHz. Capacitive transients and series resistance were compensated and linear leakage current was subtracted using the P/6 protocol (leakage current was calculated based on 6 small and short hyperpolarising pre-pulses before conditioning and test-pulses; see user manual of pClamp 5.5).

Effects on Na^+ currents were measured applying voltage steps (20 ms duration) to −10 mV from a holding potential of −70 mV in every 20 s. Drugs dissolved in bath solution were administered as single or increasing concentrations, 3 min apart, with a seven-barrelled, gravity-driven, fast drug administration system directly to the neurones. Steady-state inactivation curves were obtained by clamping the membrane at one of a series of 15-s pre-pulse potentials, followed 1 ms later by a 20-ms test pulse to −10 mV.

In patch-clamp experiments, the peak amplitudes of the Na^+ currents evoked by the voltage steps were measured using the pClamp 5.5 program. IC_{50} values were calculated for each experiment by direct curve-fitting (SigmaPlot for Windows). Shifts in steady-state inactivation curves were calculated using two-state Boltzmann fitting of the data.

2.5. Veratridine-induced cell death

Protective effects against veratridine-induced cell death were examined as described earlier (Lakics et al., 1995a,b). On day 14, in vitro, cultures were exposed to increasing concentrations of vinca derivatives in the presence and absence of 100 μM veratridine. Drugs were co-administered and dissolved in Hank's balanced salt solution. Cell death was evaluated under the phase contrast microscope and quantified by the measurement of lactate dehydrogenase leakage according to the method of Wroblewski and La Due (1955). The data were corrected for spontaneous cell death measured in the absence of veratridine and vinca derivatives. Results were expressed as percent protection, with 0 and 100% being defined as values estimated in the presence and absence of veratridine, respectively.

2.6. Maximal electric shock

Groups of 10 male NMRI outbred mice (weighing 20–25 g) were treated i.p. with five graded doses of vinca derivatives 30 min before the induction of seizures by maximal electric shock. Vehicle-treated controls were also included in each experiment. Before generating convulsions, the corneal surface was wetted with a drop of saline, then maximal electric shock (tonic hindlimb extension) was evoked bicorneally by an impulse of 60 mA, 60 Hz for 0.3 s using a shock generator (ECT Unit 7801; Ugo Basile, Italy). The seizures in control mice began with tonic hindlimb flexion turning into a tonic extension followed by the generalisation of clonic convulsions. Thereafter, a transient, post-convulsion depression and a short recovery period was observed. Protection (anticonvulsant activity) was defined as the abolition of the hindlimb

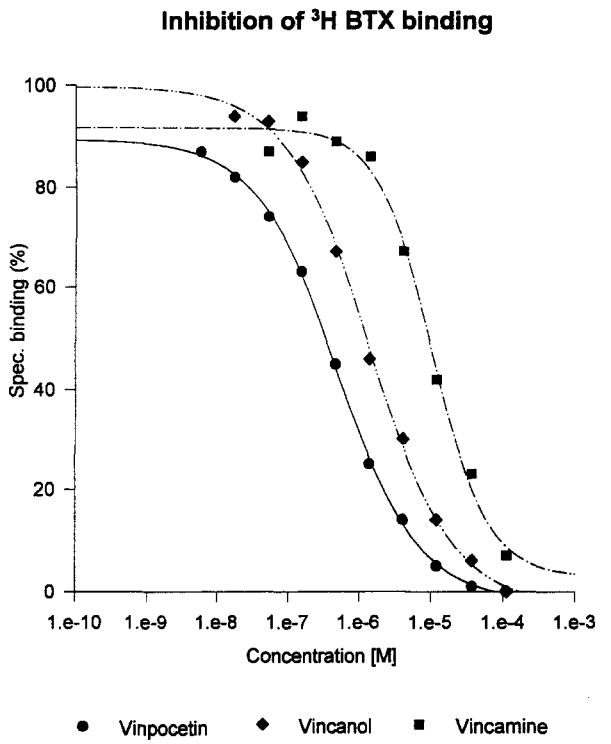

Fig. 1. Concentration-dependent displacement of [^{3}H]batrachotoxin (BTX) binding by vinca derivatives in rat cerebral synaptosomes. The following IC_{50} values were obtained (μM): vinpocetine 0.34 ± 0.07, vincanol 1.89 ± 0.27 and vincamine 10.72 ± 1.26 (mean $\pm$ S.E.M., $n = 3$–4).

extensor component of the seizures. ED_{50} values were estimated by linear regression analysis using the SigmaPlot for Windows software.

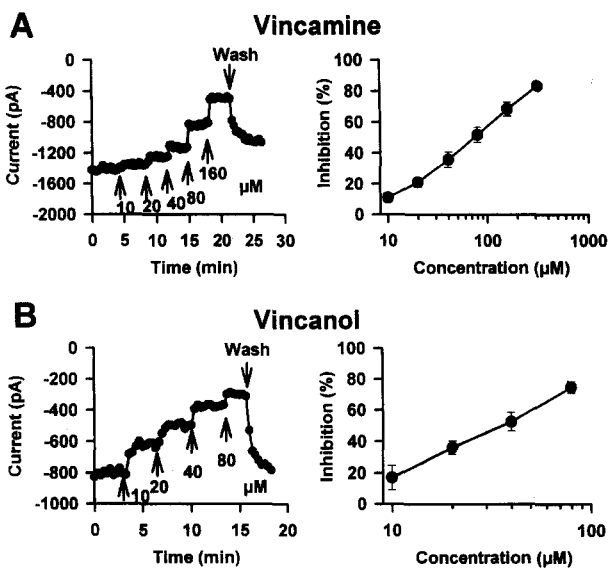

Fig. 2. Inhibition of voltage-dependent Na^+ currents by vincamine (A) and vincanol (B) in cultured rat cortical neurones. Vinca derivatives evoked a concentration-dependent inhibition of Na^+ currents evoked by voltage steps to -10 mV from a holding potential of -70 mV. Concentration-inhibition curves for vincanol and vincamine yielded IC_{50} values of 40.15 ± 1.67 and 72.1 ± 7.7 μM, respectively (mean $\pm$ S.E.M., $n = 5$).

3. Results

3.1. [^{3}H]Batrachotoxin binding

Vinpocetine, vincamine and vincanol caused a concentration-dependent inhibition of [^{3}H]batrachotoxin binding to rat cortical synaptosomes (Fig. 1). The estimated IC_{50} values were in the submicromlar and low micromolar range (see legend to Fig. 1) and revealed the following order of potencies: vinpocetine > vincamine > vincanol. Anticonvulsant Na^+ channel blockers, phenytoin (IC_{50} = 36.6 ± 6.9 μM, $n = 3$) and carbamazepine (IC_{50} 163 ± 23.6 μM, $n = 3$) were less potent to displace batrachotoxin binding than any of the vinca derivatives.

3.2. Patch-clamp experiments

In whole-cell patch-clamp experiments, vincamine and vincanol produced a concentration-dependent blockade of Na^+ currents evoked by voltage steps from -60 to -10

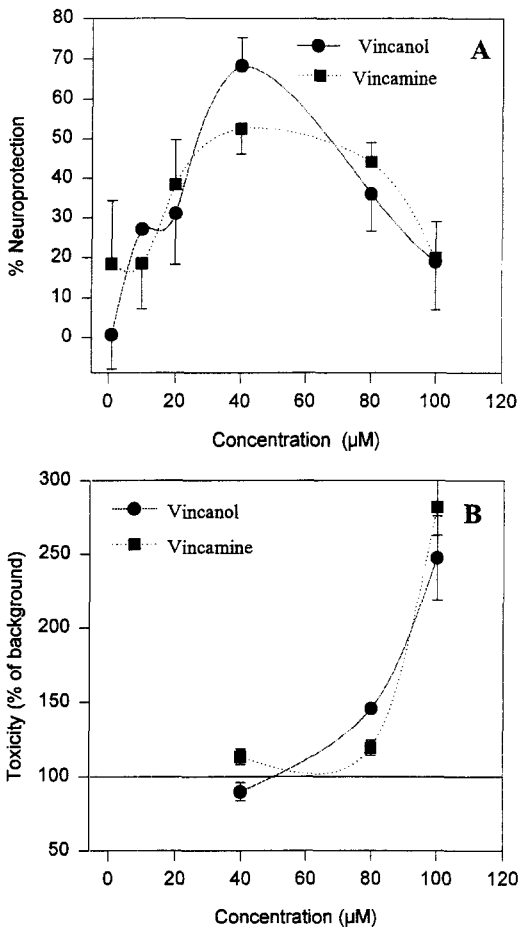

Fig. 3. Concentration-dependent protection by vincamine and vincanol against veratridine-induced cell death in cortical cultures (A). Note the bell-shaped concentration-response curves. Approximate IC_{50} values, determined on the basis of the ascending segments of the curves, were 26 and 33 μM for vincamine and vincanol, respectively. At higher concentrations, vincamine and vincanol induced cell death in a concentration-dependent manner, even in the absence of veratridine (B). Points and vertical bars represent mean $\pm$ S.E.M. values ($n = 3$). Repeat experiments gave similar results.

mV (Fig. 2). The estimated IC_{50} values were as follows: vincamine 72.1 ± 7.7 μM, vincanol 40.15 ± 1.67 μM. For comparison, vinpocetine exhibited an IC_{50} value of 44.2 ± 14.6 μM under the same experimental conditions (Molnár and Erdõ, 1995).

3.3. Veratridine-induced cell death

Vinca derivatives protected primary cortical cultures, in a concentration-dependent manner, against the cell death induced by exposure to 100 μM veratridine (Fig. 3a). The approximate IC_{50} values determined for vincamine and vincanol were 26 and 33 μM, respectively. Under the same experimental conditions, vinpocetine proved to be much more potent (IC_{50} = 0.49 μM) than vincamine and vincanol to prevent veratridine toxicity in cortical cultures (Lakics et al., 1995a). It should also be noted that, in contrast to that of vinpocetine, the protective effects of vincamine and vincanol showed bell-shaped concentration-response curves, most probably due to the toxicity of the compounds themselves at concentrations over 50 μM (Fig. 3b).

3.4. Maximal electric shock convulsions

Upon their i.p. administration, 30 min before the generation of seizures, vincamine, vincanol and vinpocetine inhibited, in dose-dependent manner, the tonic convulsions (hindlimb extension) evoked by maximal electric shock (Fig. 4). The ED_{50} values estimated for vincamine, vincanol and vinpocetine were 15.4, 14.6 and 27.2 mg/kg, respectively. Under the same conditions, phenytoin and carbamazepine exerted more pronounced anticonvulsant effects with ED_{50} values of 2.5 and 6 mg/kg, respectively (not illustrated).

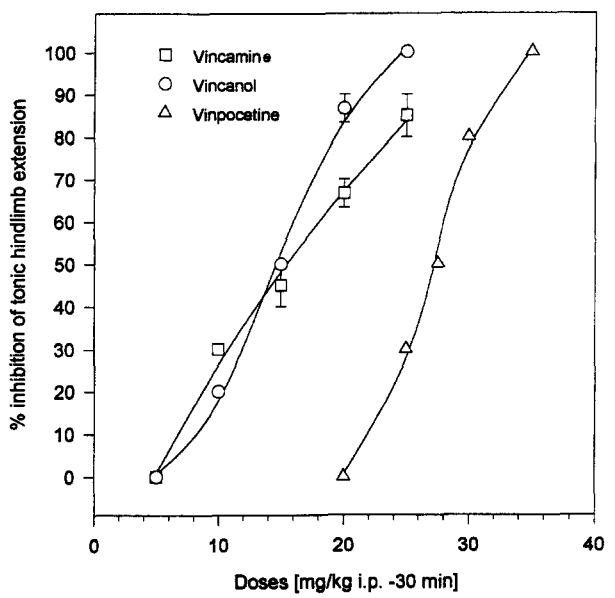

Fig. 4. Dose-dependent protection by vinca derivatives against maximal electric shock convulsions in mice. Mice were treated i.p. with graded doses of the vinca derivatives 30 min before induction of convulsions by maximal electric shock. Points without vertical bars represent percentage inhibition of tonic hindlimb extension in groups of 10 animals. Points with vertical bars refer to mean values ± S.E.M. of data obtained in 3–4 repeated experiments, each with 10 animals. The following ED_{50} values were obtained (mg/kg i.p.): vincamine 15.4, vincanol 14.6 and vinpocetine 27.2.

4. Discussion

The results presented above show that the three vinca derivatives, vinpocetine, vincamine and vincanol, are potent displacers of [^{3}H]batrachotoxin binding to voltage-gated, neuronal Na^+ channels in rat cortical synaptosomes, indicating their micromolar affinity to the target protein of the channel. Moreover, vinpocetine, vincamine and vincanol were able to block voltage-sensitive Na^+ currents in cultured cortical neurones at comparable micromolar concentrations, supporting the view that the interaction observed at the level of ligand binding has functional relevance in terms of channel blockade. The ability of the three vinca derivatives to protect cortical neurones against veratridine-induced neurotoxicity suggests that vinpocetine, vincamine and vincanol possess a neuroprotective potential that is related to the blockade of voltage-gated Na^+ channels in vitro.

The orders of potency of the three vinca derivatives differed markedly in the in vitro tests employed. The neuroprotective efficacies of the drugs did not correspond with their capacities to inhibit Na^+ currents. This phenomenon has recently been observed with a series of Na^+ channel blockers (Lakics et al., 1995b) and is proposed to result from different conformational stages of the target channel in the presence of veratridine (neuroprotection studies) and under the influence of voltage steps (patch-clamp experiments), which may influence binding probability and affinity of a particular blocker to the pharmacophore (Corbett and Vander Klok, 1994). Whatever is the case, the in vitro results now presented provide evidence that vinpocetine, vincamine and vincanol are potent blockers of voltage-gated Na^+ channels.

In laboratory rodents, the anticonvulsant drugs, phenytoin and carbamazepine, are effective against seizures evoked by maximal electic shock, an activity that is taken to result from the blockade of voltage-dependent Na^+ channels (see Levy et al., 1995). Considering that the three vinca derivatives possess a marked potency to block Na^+ channels, it seemed reasonable to examine whether these compounds may also protect against maximal electric shock convulsions. Our results show that the vinca derivatives examined do antagonise maximal electric shock-induced convulsions in a dose-dependent manner, supporting the

view that these compounds are centrally active Na^+ channel blockers. The different orders of potency of the vinca derivatives as anticonvulsants in vivo and in any of the in vitro models may reflect differences in their bioavailabilities. Alternatively, anticonvulsant efficacies of the vinca derivatives may be modulated through concomitant interactions at molecular targets other than Na^+ channels.

A growing body of recent evidence suggests that centrally active blockers of voltage-gated Na^+ channels may be beneficial not only as anticonvulsants, but also as promising neuroprotective agents against acute ischemic traumas (Taylor and Meldrum, 1995). Our finding that the well-known neuroprotective/nootropic agents vinpocetine, vincanol and vincamine are potent blockers of voltage-gated Na^+ channels supports the view that the previously recognised therapeutic efficacy of these vinca derivatives is related to an interaction at this molecular target. Moreover, the present results showed that the Na^+ channel-blocking property is not restricted to vinpocetine but may be a more general, common characteristics of certain vinca derivatives.

In summary, the present results provide evidence that the vinca derivatives vincamine, vincanol and vinpocetine are potent inhibitors of voltage-sensitive Na^+ channels, and suggest a contribution of this mechanism to the therapeutic efficacy of the drugs.

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