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Inhibition of human $Na_v1.5$ sodium channels by strychnine and its analogs

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ABSTRACT

Strychnine and brucine from the seeds of the plant *Strychnos nux vomica* have been shown to have interesting pharmacological effects on several neurotransmitter receptors. In this study, we have characterized the pharmacological properties of strychnine and its analogs on human $Na_v1.5$ channels to assess their potential therapeutic advantage in certain arrhythmias. Among the eight alkaloids, only strychnine and icajine exhibited inhibition potency on the $Na_v1.5$ channel with the half-maximum inhibition (IC_{50}) values of 83.1 μM and 104.6 μM, respectively. Structure–function analysis indicated that the increased bulky methoxy groups on the phenyl ring or the negatively charged oxygen atom may account for this lack of inhibition on the $Na_v1.5$ channel. Strychnine and icajine may bind to the channel by cation–π interactions. The substitution with a large side chain on the phenyl ring or the increased molecular volume may alter the optimized position for the compound close to the binding sites of the channel. Strychnine and icajine bind to the $Na_v1.5$ channel with a new mechanism that is different from TTX and local anesthetics. They bind to the outer vestibule of the channel pore with fast association and dissociation rates at resting state. Strychnine and icajine had little effect on steady-state fast inactivation but markedly shifted the slow inactivation of $Na_v1.5$ currents toward more hyperpolarized potentials. The property of icajine influencing slow-inactivated state of $Na_v1.5$ channel would be potential therapeutic advantages in certain arrhythmias.

1. Introduction

$Na_v1.5$, the cardiac isoform of the voltage-gated sodium channels (VGSCs), is critical to heart excitability and conduction. The $Na_v1.5$ channel protein that is in humans is encoded by the *SCN5A* gene, and the current of $Na_v1.5$ is tetrodotoxin (TTX)-resistant [1–4]. The inward sodium current (I_{Na}) through open Na_v channels during the action potential plateau will counter the effects of the increased K^+ efflux, thereby slowing or delaying repolarization and increasing action potential durations [5]. Therefore, changes in the probability that Na_v channels are open at voltages corresponding to the plateau could markedly affect action potential waveforms in ventricular cells. Voltage-dependent inactivation of $Na_v1.5$, a consequence of voltage-dependent activation, is characterized by at least two distinguishable kinetic components: an initial rapid component (fast inactivation) and a slower component (slow inactivation). Ion-channel blocking drugs that inhibit peak I_{Na} in the ventricle were associated with increased mortality in the cardiac arrhythmia suppression trail [6]. However, blockage of the slow inactivating component of I_{Na} might be a useful antiarrhythmic target [7]. This was highlighted by reports that mutations in sodium channels that produced a slowing of inactivation were responsible for some serious clinical arrhythmias [8] and that blockage of the currents is a useful therapeutic intervention in some long QT cases [9–11]. Thus, drugs that behave with a preference for the slow-inactivated state might be good candidates for finding safer sodium blockers that are useful as antiarrhythmics.

Nux vomica, the dried seed of *Strychnos nux vomica* L., which is a moderate-sized tree from the family *Loganiaceae* that is grown extensively in southern Asian countries, has been effectively used in Traditional Chinese Medicine for the treatment of blood circulatory problem and rheumatic pain [12]. Alkaloids are the main bioactive chemicals in *nux vomica* and are responsible for the pharmacological and toxic effects exerted by *nux vomica*. Strychnine and brucine, two dominating monomeric bisindole alkaloids in *nux vomica*, are deadly poisons; thus, *Nux vomica* must be processed before being used as a medicine [13]. During the processing, part of the intrinsic alkaloids, such as strychnine and

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brucine, transform into their isoforms (e.g., isostrychnine and isobrucine) or nitrogen oxidative derivatives (e.g., strychnine N-oxide and brucine N-oxide), which possess more or equally potent pharmacological effects compared to their parent compounds without having toxic side effects [14]. Additionally, some alkaloid fractions with low content, such as icajine and novacine, are extracted from the plant and have similar structures as strychnine. Together, all of these alkaloids share the common structural feature of one or more phenolic rings with VGSC-blocking drugs, such as lidocaine, which has cardioprotective efficacy [15]. Therefore, we hypothesized that these alkaloids may inhibit I_{Na} expressed specific in cardiac muscle and function as cardioprotective drugs.

In this study, strychnine and its analogs have been characterized pharmacologically on human embryonic kidney tsA201 cells that are transfected with the human $Na_v1.5$ subtype and ventricular myocytes. Among the eight alkaloids (mentioned above), strychnine and icajine exhibited inhibitory effects on the $Na_v1.5$ channel and behaved as slow-inactivated state-preferring drugs. This study provided new insight into the structure–activity relationships for the alkaloid strychnine and its analogs on the voltage-gated sodium channels.

2. Methods

2.1. Alkaloids preparation

Strychnine, brucine, strychnine N-oxide and brucine N-oxide were purchased from Sigma–Aldrich (St. Louis, MO, USA) as standards. Unless otherwise indicated, all compounds were purchased from Sigma–Aldrich. Isobrucine, isostrychnine, icajine and novacine were isolated from the seeds of *S. nux-vomica.* Briefly, the seeds of *S. nux vomica* (0.9 kg) were fried for 3 min at 235 °C in a peanut oil bath. The oil was wiped off the surface of the seeds. The fried seeds were powdered and macerated for 48 h with aqueous ammonia (conc. NH_3 H_2O:H_2O, 1:9, v/v; 1.7 L) at room temperature. The mixture was percolated with the same solution, and the percolate was concentrated to 1 L under reduced pressure. The concentration was then extracted with $CHCl_3$. The residue was repeated three times with the same method. Combined $CHCl_3$ phase was evaporated to dryness in vacuo to give a $CHCl_3$ extract. The extract was dissolved in 8% citric acid aqueous solution (aqu.), and the aqu. was adjusted to pH 9–10 with 60% Na_2CO_3. The aqu. was extracted with $CHCl_3$ five times. The combined $CHCl_3$ solution was evaporated under reduced pressure to give a residue. A portion of the residue (16 g) was subjected to column chromatography over a silica gel column, eluting with a three gradient solvent system of hexane:$CHCl_3$:EtOH:Et_2NH (10:8:0.5:0.3, 5:8:0.5:0.3, 10:8:2.5:0.3, v/v), $CHCl_3$:MeOH (10:1, 5:1, v/v), and MeOH to give five fractions (Fr. 1–Fr. 5). Each fraction was subjected to column chromatography, preparative thin-layer chromatography to give the following compounds: icajine and novacine from Fr. 1, strychnine and brucine from Fr. 2, isostrychnine from Fr. 3, strychnine N-oxide and brucine N-oxide from Fr. 4 and isobrucine from Fr. 5. The structures of separating strychnine, brucine, strychnine N-oxide and brucine N-oxide were determined by thin-layer chromatography as compared to the standards. The structures of icajine, novacine, isostrychnine and isobrucine were elucidated by analysis of 1D NMR spectroscopic data. All of these compounds were identified by high-performance gel permeation chromatography with a purity >95%.

2.2. Cell culture and transfection

Culture medium, serum, antibiotics and Lipofectamine 2000 were obtained from Invitrogen (Paisley, USA). Human embryonic kidney tsA201 cells, a simian virus (SV-40)-transformed derivative of HEK-293 cells, were maintained at 37 °C in a humidified 5% CO_2 incubator (Thermo Scientific, NC, USA) in culture medium (Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 U/ml streptomycin). Confluent cells (50–70%) were plated onto a 24-well cell culture Plate 3–4 h before transfection. The tsA-201 cells were transfected with 0.9 μg of a mix of *SCN5A* cDNA ($Na_v1.5$), *SCN1B* cDNA ($Na_v\beta1$) and *SCN2B* cDNA ($Na_v\beta2$) in equi-molar ratios, together with 0.1 μg of enhanced green florescent protein cDNA using the Lipofectamine 2000 and following the manufacturer's instructions. *SCN5A*, *SCN1B* and *SCN2B* were kind gifts from Dr. Robert S. Kass (Columbia University, New York NY, USA). Cells were trypsinized 24 h after transfection and plated on poly-D-lysine-coated coverslips for recording.

2.3. Preparation of ventricular myocytes

Animal studies were conducted in accordance with the Chinese Council on Animal Care Guidelines. Ventricular myocytes were isolated from 1-day-old Sprague-Dawley rats of either sex by enzymatic dissociation as described previously [16] and modified. Briefly, the heart was rapidly removed via thoractomy after subcutaneous injection of sodium pentobarbital. Ventricles were cut into 1–2-mm cubes and dissociated by agitation (100 rpm) at 37 °C for 10 min in Hank's solution containing 0.125% trypsin. The cellular suspensions were pelleted by centrifugation at 1000 rpm for 5 min. Cells were resuspended in culture medium, containing Dulbecco's modified Eagle's medium/Ham's F-12 medium (Invitrogen, Burlington, OH) and 10% fetal bovine serum (Gibco, USA), and stand for 90 min, then shifted on to 12 mm circle coverslips in 24-well plate. Plate was maintained in a humidified incubator containing 5% CO_2, 95% air at 37 °C and culture medium was refreshed daily. The ventricular myocytes growing for 48 h on glass coverslips were used for electrophysiological studies.

2.4. Electrophysiology

$Na_v1.5$ currents were recorded from tsA-201 cells with green fluorescent signal. Whole-cell voltage-clamp recording was performed with the Axopatch 200B (Axon Instruments, Foster City, CA, USA). All voltage protocols were applied using pCLAMP 8 software (Axon) and a Digidata 1322A (Axon). Currents were amplified, low pass filtered (2 kHz) and sampled at 10 kHz. Recording patch pipettes were fire-polished to a resistance of 3–5 MΩ. For tsA201 cells recording, the internal solution contained (mM): CsCl 135, NaCl 10, and HEPES 5, adjusted to pH 7.2 with CsOH, and the bath solution contained (mM): NaCl 50, $MgCl_2$ 1, $CaCl_2$ 1.8, CsCl 5, KCl 5, D-Glucose 25, Tetraethylammonium-Cl 70, and HEPES 5, adjusted to pH 7.4 with NaOH. For ventricular myocytes recording, the internal solution contained (mM): CsF 120, NaCl 10, MgATP 5, EGTA 10, Glucose 11, and HEPES 10, adjusted to pH 7.2 with CsOH, and the bath solution contained (mM): NaCl 30, Choline chloride 110, $MgCl_2$ 1, KCl 5.4, NaH_2PO_4 0.33, and HEPES 10, adjusted to pH 7.4 with NaOH. Compounds were prepared as 100 mM stock solutions in dimethyl sulfoxide (DMSO) (Sigma) and diluted to the desired concentration in perfusion solution. The maximum DMSO concentration used was 0.5% and had no effect on current amplitude. All experiments were performed at room temperature (22–25 °C). Series resistance was compensated to 80%, with a 10 μs lag time. Data were leak-subtracted on-line using a P/4 protocol and analyzed using pClamp V8.0 (Axon).

2.5. Data analysis

Experimental data were acquired and analyzed by the programs Clampfit 8.0 (Axon) and Sigmaplot (Sigma, USA). All data are