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Tetrahydroberberine blocks ATP-sensitive potassium channels in dopamine neurons acutely-dissociated from rat substantia nigra pars compacta

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ABSTRACT

Tetrahydroberberine (THB) exhibits neuroprotective effects but its targets and underlying mechanisms are largely unknown. Emerging evidence indicates that ATP-sensitive potassium (K_{ATP}) channels in the substantia nigra pars compacta (SNc) promote Parkinson disease (PD) pathogenesis, thus blocking K_{ATP} channels may protect neurons against neuronal degeneration. In the present study, we tested a hypothesis that THB blocks K_{ATP} channels in dopaminergic (DA) neurons acutely dissociated from rat SNc. Using perforated patch-clamp recording in current-clamp mode, the functional K_{ATP} channels can be opened by persistent perfusion of rotenone, an inhibitor of complex I of the mitochondrial respiratory chain. Bath-application of THB reversibly blocks opened K_{ATP} channels in a concentration-dependent manner, which is comparable to a classical K_{ATP} channel blocker, Tol. Compared to THB analogs, *l*-stepholidine (*l*-SPD) or *l*-tetrahydropalmatine (*l*-THP), THB exhibits more profound blockade in K_{ATP} channels. In addition, exposure of THB alone to the recorded neuron increases action potential firing, and THB also restores rotenone-induced membrane hyperpolarization in the presence of dopamine D2 receptor antagonist (sulpiride), suggesting that THB exhibits an excitatory effect on SNc DA neurons through the block of K_{ATP} channels. Collectively, the blockade of neuronal K_{ATP} channels by THB in SNc DA neurons is a novel pharmacological mechanism of THB, which may contribute to its neuroprotective effects in PD.

1. Introduction

Tetrahydroberberine (THB) is a compound isolated from the Chinese herb. *l*-tetrahydropalmatine (*l*-THP) and *l*-stepholidine (*l*-SPD) are the homologues analogs of THB, and they are called terahydroprotoberberine analogs (THPBs). Accumulating lines of evidence indicate that THPBs exhibit dopamine receptor antagonistic effects on sedation, hypnosis, antinociception, anti-schizophrenia, anti-hypertension, as well as the prevention of drug addiction (Bian et al., 1986; Chu et al., 2008; Xiong et al., 1987; Zhang et al., 1986). In addition, the morphological and biochemical experiments have demonstrated that THPBs also have neuroprotective effects (Tang et al., 1999). For instance, in transient ischemic rat models, *l*-SPD antagonized ischemic injury by eliminating the activation of calcium/calmodulin-dependent protein kinase II (CCDPKII) (Tang et al., 1999), which has been reported to be involved in the mechanism of neuronal protection against ischemia (Uno et al., 1999). Furthermore, *l*-SPD also inhibited the release of lactate dehydrogenase (LDH), an indicator of injury, from neurons following ischemia, suggesting that *l*-SPD is able to decrease neuronal injury induced by hypoxia. Histological examination confirmed that *l*-SPD protects striatal cells against transient cerebral ischemic injury (Tang et al., 1999). Furthermore, the neuroprotective effects of *l*-SPD may be related to its ability to scavenge hydroxyl free radicals (Jin et al., 2000). However, the targets and underlying mechanisms of THPB-induced neuroprotection still remain elusive.

Although extensive works have indicated that DA receptors (D1 and D2) are the main targets that mediate pharmacological effects of THPBs (Chen et al., 1986, 1985; Fu et al., 2004; Hu et al., 1992; Jin et al., 1992; Shi et al., 1984; Sun and Jin, 1992; Tang et al., 1999; Zhang et al., 1999, 1998; Zhu et al., 2000) other targets also have been reported to mediate THPBs' effects including α-adrenergic receptor (Liu et al., 1989), 5-TH receptor (Miao et al., 1991), Ca^{2+} channels (Li et al., 1995; Miao et al., 1991; Shen et al., 1991) and K^+ channels (Wu et al., 1996; Wu and Jin, 1997a,b). These lines of evidence suggest that THPBs may act on multiple targets to exert their pharmacological roles in the CNS. Emerging evidence indicates that ATP-sensitive potassium (K_{ATP}) channels in the midbrain

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substantia nigra compacta (SNc) DA neurons promote pathogenesis in Parkinson disease (PD) animal models, which suggests that the blockade of K_{ATP} channels may protect neurons against MPTP neurotoxicity (Liss et al., 2005).

In the present study, we tested whether or not THB, *l*-SPD or *l*-THP blocks K_{ATP} channels in DA neurons acutely dissociated from rat SNc. Our results demonstrate that THB is a potent K_{ATP} channel blocker.

2. Materials and methods

2.1. Single DA neuron dissociation from rat SNc

The protocol for preparation of single neurons from the rat SNc was approved by the Institutional Animal Care and Use Committee of the Barrow Neurological Institute.

Single DA neurons were acutely dissociated from the SNc of 2–3-week-old Wistar rats following the protocol as previously described (Wu et al., 2004, 2006; Yang et al., 2009). Briefly, rats were anesthetized with isoflurane, and brain tissue was rapidly removed and immersed in cold (2–4 °C) dissection solution which contained: 136.7 mM NaCl, 5 mM KCl, 0.1 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 9.84 mM HEPES, 16.6 mM D-glucose, 21.9 mM sucrose, pH 7.3, 330 mOsm, oxygenated with 100% O_2 (Ishihara et al., 1995). Three 400-μm coronal slices containing the SNc were cut using a vibrotome (Vibroslice 725M, WPI, Sarasota, FL). After cutting, slices were continuously bubbled with 95% O_2–5% CO_2 at room temperature (22 ± 1 °C) for at least 1 h in artificial cerebrospinal fluid (ACSF), which contained: 124 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 1.3 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.4 mM $CaCl_2$, and 10 mM glucose, pH 7.4. Thereafter, slices were treated with pronase (1 mg per 6 ml) at 31 °C for 30 min in ACSF. The SNc was identified in a coronal slice using a stereo microscope with reference to the rat brain atlas (Paxinos and Watson, 1998), and was micro-punched out from slices using a well-polished needle. One punched piece was then transferred to a 35-mm culture dish filled with well-oxygenated standard extracellular solution, which contained: 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose 10, and 10 mM HEPES, pH 7.4 (with Tris–base). The punched piece was then dissociated mechanically using a fire-polished micro-Pasteur pipette under an inverted microscope (Olympus IX-70, Lake Success, NY). The separated cells adhered to the bottom of the culture dish within 30 min. In the present study, we used only DA neurons that maintained their original morphological features of polygonal, large or medium somata with 2–4 thick primary dendritic processes.

2.2. Perforated patch-clamp whole-cell recordings

Perforated patch whole-cell recording techniques were employed as previously described (Wu et al., 2004, 2006; Yang et al., 2009). Pipettes (3–5 MΩ) used for perforated patch recording were filled with intracellular recording solution containing 140 mM potassium gluconate, 10 mM KCl, 5 mM $MgCl_2$, and 10 mM HEPES, pH 7.2 (with Tris–OH). The amphotericin B was freshly prepared to 200–240 μg/ml from a 40 mg/ml in DMSO stock. The liquid-junction potential was 14 mV calculated using Clamplex 9.2 (Axon Instruments, Foster City, CA) and corrections were made for junction potentials post-hoc. After tight seal (>2 GΩ) formation, it usually took about 5–20 min to convert to perforated patch mode, and an access resistance of 20–60 MΩ was accepted to start the experiments. Series resistance was not compensated in this study. To measure the resting membrane potentials for pharmacological experiments, we expanded time scale and measured membrane potential at the point just before membrane hyperpolarization during drug (e.g., rotenone) exposure. We define this as the resting membrane potential level (see example in Supplemental Fig. 4). The data were filtered at 2 kHz, acquired at 10 kHz and digitized online (Digidata 1322 series A/D board, Axon Instruments, Foster City, CA). All data were displayed and stored on a PC computer. Drug application was performed using a computer-controlled "U-tube" system as previously described (Wu et al., 2004, 2006; Yang et al., 2009). All experiments were performed at room temperature (22 ± 1 °C). To enable identification of single, dissociated SNc neurons after a patch-clamp recording session, the recording pipette was filled with a fluorescent dye (Lucifer yellow CH, Sigma Chemical Co., St. Louis, MO, 1.0 mg/ml in the recording electrode) in some experiments. After conversion from the perforated patch to the conventional whole-cell recording mode, the dye was ejected into the cytoplasm by a pulse (200 ms, 0.5 Hz) of hyperpolarizing current (1.0 nA) for 3 min. Labeled cells were visualized using epifluorescence microscopy.

2.3. Immunocytochemical staining

Dissociated SNc neurons were fixed with 4% paraformaldehyde for 15 min, rinsed three times with PBS, and treated with Saponin (1 mg/ml) for 5 min to permeabilize the cells. After rinsing four times with phosphate-buffered saline, the neurons were incubated at room temperature in primary antibody TH (AB152, Chemicon International, Temecula, CA) diluted 1:1000 in Hank's balanced salt solution supplemented with 5% bovine serum albumin as a blocking agent for 30 min. Following another three rinses with phosphate-buffered saline, the secondary antibody (anti-mouse IgG cy3 conjugate, Sigma Chemical Co., St. Louis, MO) was applied at room temperature for 30 min (diluted 1:100). After rinsing a final three times with phosphate-buffered saline, the labeled cells were visualized using fluorescence microscopy.

2.4. Chemicals and statistics

Pronase was purchased from Calbiochem–Novabiochem Co (La Jolla, CA, USA); rotenone, tolbutamide (Tol), and Lucifer yellow were purchased from Sigma (St. Louis, MO, USA). THB, *l*-THP and *l*-SPD (Supplemental Fig. 1) were the gifts from Professor Guozhang Jin (Shanghai Institute of Materia Medica, P.R. China). Tetrahydroberberine (THB), isolated from a semi-synthized product with the Chinese herbs, exhibits a variety of pharmacological effects on the central nervous system (CNS). All other chemicals were purchased from Tocris Cookson, Inc. (Ballwin, MO, USA). Differences in altered membrane potentials (mV) were tested by Student's paired two-tailed *t* test using the raw data. Numerical values are presented as the mean ± SEM. The probability values of $p < 0.05$ were considered significant.

3. Results

3.1. Identification of dissociated SNc DA neurons

TH staining showed that the dissociated neurons from SNc exhibited TH positive (Fig. 1Ab, d) and negative (Fig. 1Ad*) reactions. For patch-clamp recording, DA neurons were identified early in the recording session based on previously described criteria (Lacey et al., 1989): (1) 1–3 Hz spontaneous action potential firing (Fig. 1Ba), (2) the duration of action potential is longer than 2.5 ms (Fig. 1Bb), (3) spontaneous action potential firing is eliminated by 10 μM DA (Fig. 1Ba), and (4) expression of a hyperpolarization-induced current (Fig. 1C). In some experiments, after patch-clamp recording, the fluorescence dye, Lucifer yellow (0.5 mg/ml) was delivered into recorded cell and labeled cell was stained with TH for further confirmation of DA neuronal phenotype (Supplemental Fig. 2).

3.2. Effects of the THB on functional K_{ATP} channels in SNc DA neurons

Under physiological conditions, the K_{ATP} channels are mostly closed. However, in the acutely-dissociated single neurons from rat SNc, there is background opening of K_{ATP} channels (Wu et al., 2006). To open these K_{ATP} channels, an inhibitor of complex I of the mitochondrial respiratory chain, rotenone (1 μM) was bath-applied to patch-recorded neuron under current-clamp recording mode. The opening of functional K_{ATP} channels was evident as a gradual reduction of action potential firing and hyperpolarization of membrane potential (Fig. 2A). In 30 neurons tested, the averaged resting membrane potential was −46.1 ± 0.9 mV, while after perfusion of 1 μM rotenone for 1–3 min, the membrane potential was hyperpolarized to −61.1 ± 0.9 mV ($p < 0.001$). In the presence of rotenone, the application of a classical K_{ATP} channel blocker, Tol (100 μM) restored membrane potential hyperpolarization and fired action potential (Fig. 2A), suggesting an opening of K_{ATP} channels by rotenone. Alternatively, the functional K_{ATP} channels were also able to be opened by a K_{ATP} channel opener, dizoxide (300 μM, Fig. 2B). Then, we tested the effects of the THB on theses opened K_{ATP} channels. As shown in Fig. 2C, bath-perfusion of 100 μM THB increased firing rate of spontaneous action potential firing with a moderate membrane potential depolarization. Before and after exposure to 100 μM THB, the values of fairing rate were 1.4 and 2.2 Hz, respectively ($p < 0.05$, $n = 7$); and membrane potentials were −46.5 ± 0.9 and −41.3 ± 1.9 mV, respectively ($p < 0.05$, $n = 7$). Whereas a classical K_{ATP} channel blocker Tol (100 μM) showed little depolarization of membrane potential (from −44.4 ± 1.6 to −44.1 ± 1.8 mV, $p > 0.05$, $n = 5$). In the presence of rotenone, THB restored membrane potential hyperpolarization, which was comparable to 100 μM Tol (Fig. 2D). During persistent perfusion of 1 μM rotenone, THB depolarized