Toxicology in Vitro 25 (2011) 1644–1653

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Effects of Resibufogenin and Cinobufagin on voltage-gated potassium channels in primary cultures of rat hippocampal neurons

Shuang Hao [a], Yong-Ming Bao [a], Li-Jia An [a], Wei Cheng [b], Rong-Guo Zhao [c], Jing Bi [a], He-Shuang Wang [d], Chang-Sen Sun [b], Ji-Wen Liu [a], Bo Jiang [a,*]

[a] School of Life Science and Biotechnology, Dalian University of Technology, Dalian, Liaoning 116024, PR China
[b] School of Physics and Optoelectronic Technology, Dalian University of Technology, Dalian, Liaoning 116024, PR China
[c] Guoren Biological Technology Co. Ltd., Dalian, Liaoning 116023, PR China
[d] Center of Biotherapy, 255 Hospital of Chineses People's Liberation Army, Tangshan, Heibei 063000, PR China

ARTICLE INFO

Article history:
Received 14 December 2010
Accepted 4 July 2011
Available online 26 July 2011

Keywords:
Resibufogenin (RBG)
Cinobufagin (CBG)
Delayed rectifier potassium current (I_K)
Transient potassium current (I_A)
Hippocampal neurons
Patch clamp

ABSTRACT

Outward delayed rectifier potassium channel and outward transient potassium channel have multiple important roles in maintaining the excitability of hippocampal neurons. The present study investigated the effects of two bufadienolides, Resibufogenin (RBG) and Cinobufagin (CBG), on the outward delayed rectifier potassium current (I_K) and outward transient potassium current (I_A) in rat hippocampal neurons. RBG and CBG have similar structures and both were isolated from the venom gland of toad skin. RBG inhibited both I_K and I_A, whereas CBG inhibited I_K without noticeable effect on I_A. Moreover, at 1 μM concentration both RBG and CBG could alter some channel kinetics and gating properties of I_K, such as steady-state activation and inactivation curves, open probability and time constants. These findings suggested that I_K is probably a target of bufadienolides, which may explain the mechanisms of bufadienolides' pathological effects on central nervous system.

1. Introduction

Resibufogenin (RBG, $C_{24}H_{32}O_4$) and Cinobufagin (CBG, $C_{26}H_{34}O_6$) are two bufanolide steroid compounds isolated from the secretion (known as Chansu, which has been used as a traditional Chinese medicine) of the Asiatic toad *Bufo gargarizansa* Cantor or *Bufo melanostictus* Schneider. RBG, CBG and other bufanolide steroid compounds, collectively known as bufadienolides, have similar chemical structures to digitoxigenin (Fig. 1) and possess both pharmacological and toxicological effects as shown by *in vitro* and *in vivo* studies such as cardiotoxic, cardiotonic, anesthesia, and anti-tumor effects (Xie et al., 2000; Krylov, 2002). The effects of Chansu formulations on the electrophysiological characteristics of membrane, especially that of mammalian myocardial muscle cells have been investigated, and some researchers have demonstrated that RBG exerts its pharmacological and toxicological effects on cardiac cells through regulating potassium rectifier current (Xie et al., 2000), membrane depolarization (Xie et al., 2001) and Na^+–K^+ ATPase activity (Bick et al., 2002). All the evidences from electrophysiological experiments have revealed that ion channels might be a target of RBG in cardiac cells. In addition, a large number of ion channels in the membranes of neurons are targets for many kinds of toxins and drugs, and many neuronal protections and damages of central nervous system (CNS) are caused by improving or disrupting the functions of ion channels (Calavresi et al., 1995; Taylor and Meldrum, 1995; Deng et al., 2005, 2008; Liu et al., 2009). Meanwhile, most of the bufadienolides also have some pharmacological effects on the CNS, similar to respiratory stimulation, vasopressor action and anesthesia (Xie et al., 2001). All of these imply that ion channels may be one of the main targets of bufadienolides in the nervous system. However, whether the pharmacological effects of RBG and CBG are connected to the ion channels remain controversial, and no information on the effect of RBG or CBG on the ion channels of neurons has been reported.

Voltage-gated potassium (kV) channels are recognized as important determinants that maintain neuronal excitability and function (Alshuaib and Mathew, 2002) via regulating resting membrane potential (RP), interspike membrane potential, and spike frequency (Rudy, 1988; Pongs, 1999). The kV currents recorded in rat

Abbreviations: RBG, Resibufogenin; CBG, Cinobufagin; kV channels, voltage-gated potassium channels; I_K, outward delayed-rectifier potassium currents; I_A, outward transient potassium currents; CNS, central nervous system; RP, resting potential; AP, action potential; DMEM/F12, Dulbecco's modified Eagle's medium/nutrient F12; TTX, tetrodotoxin; TEA-Cl, tetraethylammonium chloride; *Po*, open probability.

* Corresponding author. Tel.: +86 411 84706356; fax: +86 411 84706365.
E-mail address: bjdlut@yahoo.com.cn (B. Jiang).

0887-2333/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2011.07.001

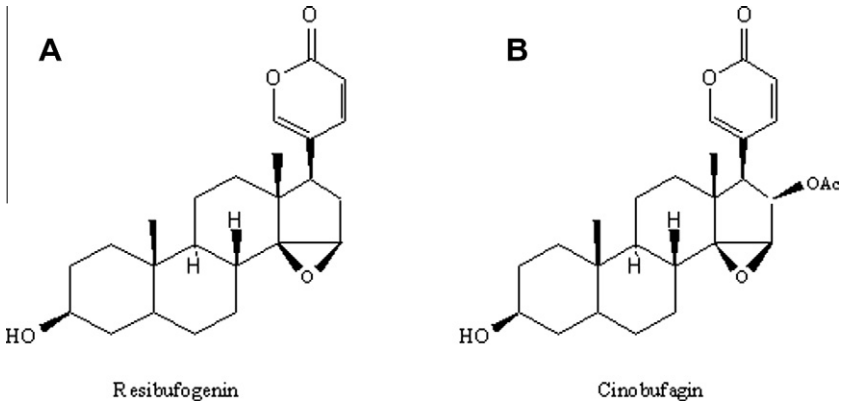

Fig. 1. Structures of RBG & CBG.

hippocampal pyramidal neurons are classified into four components, based on their specific voltage dependence of inactivation, and their dependence on calcium influx. The four currents include three kinds of outward potassium currents (outward delayed rectifier current, transient or "A-type" current, Ca^{2+}-dependent current of low and high conductance) and one inward rectifier potassium current (Storm, 1990; Vreugdenhil et al., 1995). It is well known that delayed rectifier potassium current (I_K) and transient outward potassium current (I_A) are essential for controlling and modulating the action potential (AP) in various excitable cells such as atrial myocytes, sympathetic neurons, and hippocampal neurons (Tessier et al., 1999; Malin and Nerbonne, 2002; Zhang et al., 2004; Tian et al. 2009), producing a series of physiological and pathological changes.

In the present study, the influences of RBG and CBG on the I_K and I_A of cultured hippocampal neurons were investigated by using whole-cell and single-channel patch clamp recordings, and the possible mechanisms by which these two bufadienolides exert their effects on I_K and I_A were also explained.

2. Materials and methods

2.1. Cell culture

Primary hippocampal CA1 pyramidal neurons were isolated from the brain of 24-h neonatal SD rats (Experimental Animal Center, Dalian Medical University, China). All experiments were conducted in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all efforts were made to minimize the number of animals used and their suffering.

Briefly, newborn SD rats ($n = 20$) were decapitated to obtain the brains. After removing the blood vessels and piamater, the hippocampi were quickly separated, cut into 400-μm slices and incubated with 0.125% trypsin in PBS at 37 °C for 30 min, followed by dispersion via mechanical dissociation in culture medium. The suspension was then placed on 0.01% poly-D-lysine-coated glass slip inside a 35-mm plate at a density of approximately 2×10^5 cells/ml. The culture medium consisted of Dulbecco's modified Eagle's medium/nutrient F12 (DMEM/F12) supplemented with 10% (v/v) heated-inactivated fetal bovine serum, 50 U/ml penicillin, 1.2 g/l sodium bicarbonate and 2 mmol/L L-glutamine. Cultures were maintained in a humidified incubator with 95% O_2 and 5% CO_2 at 37 °C. After 24 h of incubation the medium was replaced with fresh one and incubated for another 24 h, followed by addition of 10 μM arabinoside cytosine. Half of the culture medium was removed and replaced with fresh medium at every 48 h thereafter. All experiments were carried out within the 5th–10th day after plating.

2.2. Electrophysiological recordings

Conventional whole-cell patch-clamp recordings and single-channel patch-clamp recordings were performed with primary cultured hippocampal neurons under standard configuration (Houzen and Kanno, 1998). The recording pipettes were prepared from borosilicate glass using a two-stage puller (PC-10, Narishsige, Tokyo, Japan) to gain approximately 1–2 μm tip diameter. Patch pipettes had a tip resistance of 5–10 MΩ when filled with pipette solution at pH 7.2 containing (in mM): KCl (65), KF (80), KOH (5), HEPES (10), EGTA (10), and Na_2-ATP (2). The neurons were transferred to a 1.0-ml recording chamber mounted on an inverted microscope (IX 71, Olympus) and immersed in the standard bath solution at pH 7.2 containing (in mM): NaCl (150), KCl (5), $MgCl_2$ (1.1), $CaCl_2$ (2.6), HEPES (10), and Glucose (10). Recordings were performed with an EPC-10 (HEKA, Germany) amplifier driven by PULSE software.

For the whole-cell recording, the membrane was ruptured with gentle suction to obtain the whole-cell patch-clamp configuration after a giga ohm seal (>1 GΩ) formation. During the recording process, leak subtraction was compensated for by using a P/4 protocol. Capacitance and series resistance were routinely compensated by 60–80% in all experiments. Signals were low-pass filtered at 2 kHz and digitized at 10 kHz. For recording voltage-gated potassium current, tetrodotoxin (TTX, 1 μM) and $CdCl_2$ (0.3 mM), were added into the bath solution to block voltage-dependent Na^+ and Ca^{2+} currents, respectively.

For the single-channel patch-clamp, the pipettes were filled with the pipette solution at pH 7.3 containing (in mM): NaCl (130), KCl (3), $MgCl_2$ (2), $CaCl_2$ (2), HEPES (10). After a giga ohm seal (>1 GΩ) formation, the pipette was raised gently and rapidly to obtain the inside–out configuration, then the standard bath solution was replaced with single-channel bath solution at pH 7.3 containing (in mM): KCl (130), $MgCl_2$ (2), EGTA (2), HEPES (10) and Glucose (10). Signals were low-pass filtered at 2 kHz and digitized at 20 kHz. All experiments were performed at room temperature (22–25 °C).

2.3. Reagents

RBG and CBG (Purity ⩾98%, Fig. 1B), purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China), were dissolved in dimethyl sulfoxide (DMSO) as stock solutions and stored at −20 °C. The stock