Life Sciences 88 (2011) 898–907

Contents lists available at ScienceDirect

Life Sciences

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

Mechanism underlying the block of human $Ca_v3.2$ T-type Ca^{2+} channels by benidipine, a dihydropyridine Ca^{2+} channel blocker

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ARTICLE INFO

Article history:
Received 30 March 2010
Accepted 26 March 2011

Keywords:
$Ca_v3.2$
T-type Ca^{2+} channel
Benidipine
Ca^{2+} channel blocker
HEK-293 cells
Whole-cell patch-clamp
Ca^{2+} mobilization

ABSTRACT

Aims: Benidipine, a dihydropyridine Ca^{2+} channel blocker, has been reported to block T-type Ca^{2+} channels; however, the mechanism underlying this effect was unclear. In this study, we characterized the mechanism responsible for this blocking activity. Furthermore, the blocking activity was compared between two enantiomers of benidipine, (S, S)- and (R, R)-benidipine.
Main methods: Human $Ca_v3.2$ ($hCa_v3.2$) T-type Ca^{2+} channels stably expressed in the human embryonic kidney cell line, HEK-293, were studied in whole-cell patch-clamp recordings and Ca^{2+} mobilization assay.
Key findings: In whole-cell patch-clamp recordings, benidipine blocked $hCa_v3.2$ T-type Ca^{2+} currents elicited by depolarization to a comparable extent as efonidipine. The block was dependent on stimulation frequency and holding potential, but not test potential. Benidipine significantly shifted the steady-state inactivation curve to the hyperpolarizing direction, but had no effect on the activation curve. Benidipine prolonged the recovery from inactivation of $hCa_v3.2$ T-type Ca^{2+} channels without any effect on the kinetics of activation, inactivation, or deactivation. In the Ca^{2+} mobilization assay, benidipine was more potent than efonidipine in blocking Ca^{2+} influx through $hCa_v3.2$ T-type Ca^{2+} channels. (S, S)-Benidipine was more potent than (R, R)-benidipine in blocking $hCa_v3.2$ T-type Ca^{2+} currents, but there was no difference in blocking the Ca^{2+} influx.
Significance: We have characterized the blocking activity of benidipine against $hCa_v3.2$ Ca^{2+} channels and revealed the difference between the two enantiomers of benidipine. The blocking action of benidipine could be mediated by stabilizing $hCa_v3.2$ Ca^{2+} channels in an inactivated state.

Introduction

Voltage-gated Ca^{2+} channels can be functionally classified into different subtypes (L-, N-, P/Q-, R-, and T-type) based on their electrophysiological and pharmacological properties (Catterall, 2000; Perez-Reyes, 2003; Catterall et al., 2005). T-type Ca^{2+} channels show unique biophysical properties, including low voltage activation, transient inactivation kinetics, and low conductance of Ba^{2+}, which are absent in other voltage-gated Ca^{2+} channels. Three different α1 subunits of T-type Ca^{2+} channels have been identified and cloned: $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$ (Catterall, 2000; Perez-Reyes, 2003; Catterall et al., 2005). $Ca_v3.1$ is expressed in the brain and heart. $Ca_v3.2$ is ubiquitously expressed in the kidney, heart, adrenal cortex, liver and brain. $Ca_v3.3$ is exclusively expressed in the brain. T-type Ca^{2+} channels have been implicated in cardiac pacemaker activity, renal efferent arteriolar tone, aldosterone production, neuronal excitability, and nociception (Perez-Reyes, 2003; Ono and Iijima, 2005; Vassort et al., 2006; Hayashi et al., 2007; Shin et al., 2008). T-type Ca^{2+} channel blockers might therefore be potentially useful in the treatment of cardiovascular disease, chronic kidney disease, epilepsy, and pain.

Benidipine, a dihydropyridine Ca^{2+} channel blocker, elicits vasodilatation of coronary and peripheral arteries by blocking L-type Ca^{2+} channels (Yao et al., 2006). In addition, benidipine causes dilation of renal efferent arterioles and inhibition of aldosterone production, which cannot be explained by the block of L-type Ca^{2+} channels. (Kawata et al., 1997; Morikawa et al., 2002; Uzu et al., 2007; Akizuki et al., 2008). These pharmacological effects appear to be due to the additional block of T-type Ca^{2+} channels. It has been demonstrated that benidipine blocks T-type Ca^{2+} channels expressed in Xenopus oocytes (Furukawa et al., 2005, 2009) or endogenous T-type Ca^{2+} channels in the human adrenocortical cell line NCI-H295R (Akizuki et al., 2008). However, the precise mechanism by which benidipine blocks T-type Ca^{2+} channels still remains unclear, in spite of the fact that the pharmacological and therapeutical

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0024-3205/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2011.03.019

profiles of the drugs that target ion channels are dependent on the mechanism of ion channel block (Triggle, 1999). Furthermore, although benidipine is a racemic mixture consisting of (S, S)- and (R, R)-benidipine, whether the blocking activity against T-type Ca^{2+} channels differs between the two enantiomers of benidipine remains to be determined.

In this study, the blocking activity of benidipine on human $Ca_v3.2$ ($hCa_v3.2$) T-type Ca^{2+} channels stably expressed in the human embryonic kidney cell line, HEK-293, was characterized using whole-cell patch-clamp recordings and Ca^{2+} mobilization assay. The blocking activity against $hCa_v3.2$ T-type Ca^{2+} channels was also compared between (S, S)- and (R, R)-benidipine.

Materials and methods

Materials

Benidipine, (S, S)-benidipine, (R, R)-benidipine, and amlodipine were synthesized in our laboratories as described (Arrowsmith et al., 1986; Muto et al., 1988). Efonidipine was extracted from tablets (Zeria, Tokyo, Japan). The purity for all of the compounds was over 99%. The following materials were purchased from the suppliers as indicated: nifedipine (Sigma-Aldrich, Saint Louis, MO, USA); mibefradil (Sigma-Aldrich); and pimozide (Tocris Bioscience, Ellisville, MO, USA). All compounds were dissolved in DMSO and stored at −20 °C before use. The final concentration of DMSO in the assay systems described below was 0.01 or 0.1% v/v. The chemical structures of the dihydropyridine Ca^{2+} channel blockers are illustrated in Fig. 1.

Construction of expression vectors

The plasmid construct encoding the $hCa_v3.2$ T-type Ca^{2+} channel was purchased from OriGene (Rockville, MA, USA). A DNA fragment encoding the $hCa_v3.2$ cDNA was enzymatically excised and cloned into pcDNA3.1(+)/Hygro (pcDNA3.1-$hCa_v3.2$).

Cell culture and generation of cell lines stably expressing $hCa_v3.2$ T-type Ca^{2+} channels

HEK-293 cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. For generation of cell lines stably expressing $hCa_v3.2$ T-type Ca^{2+} channels, pcDNA3.1-$hCa_v3.2$ was transfected into HEK-293 cells using the FuGENE 6 transfection reagent (Roche, Basel, Switzerland). The transfected cells were selected with 300 μg/mL hygromycin. Hygromycin-resistant cells were obtained by limited dilution and then were screened for $hCa_v3.2$-expressing clones by the Ca^{2+} mobilization assay described below.

Electrophysiology

HEK-293 cells stably expressing $hCa_v3.2$ T-type Ca^{2+} channels (HEK-293/$hCa_v3.2$ cells) were dissociated by digestion with 0.25% trypsin plus 1 mM EDTA, seeded on 35-mm culture dishes containing 12-mm diameter glass coverslips at a density of 1.5×10^4 cells/dish, and then cultured for one to three days in culture medium before electrophysiological recordings. The dishes were positioned on the stage of an inverted microscope and superfused with a bath solution (140 mM NaCl, 2 mM $CaCl_2$, and 10 mM HEPES, adjusted to pH 7.4 with NaOH). Membrane currents in HEK-293/$hCa_v3.2$ cells were recorded using the conventional whole-cell patch-clamp technique. Patch pipettes were fabricated from glass capillary tubes (GDC1.5; Narishige, Tokyo, Japan) by a vertical two-stage electrode puller (PP-83; Narishige). Patch pipettes had a resistance of 3–5 MΩ when filled with a pipette solution (130 mM KCl, 5 mM Mg-ATP, 11 mM EGTA, and 10 mM HEPES, adjusted to pH 7.4 with KOH). The resting membrane potential of HEK-293/$hCa_v3.2$ cells was recorded in the whole-cell configuration immediately after breaking the patch membrane with an external medium made of the Ca^{2+} mobilization assay buffer, to be described in the next section. Membrane currents and potential were recorded with an Axopatch 1D (Axon Instruments, Inc., Foster City, CA, USA) and the pClamp6 data acquisition software package (Axon Instruments, Inc.). The sampling frequency for acquisition was 10 kHz. Data were filtered at 1 kHz. All experiments were performed at room temperature (22–25 °C) under protection from light. Capacitance and series resistance were compensated by 80% without oscillations. Leakage was subtracted, based on linear interpolation between the current at the holding potential and 0 mV. All compounds were added to the bath solution and applied by continuous superfusion.

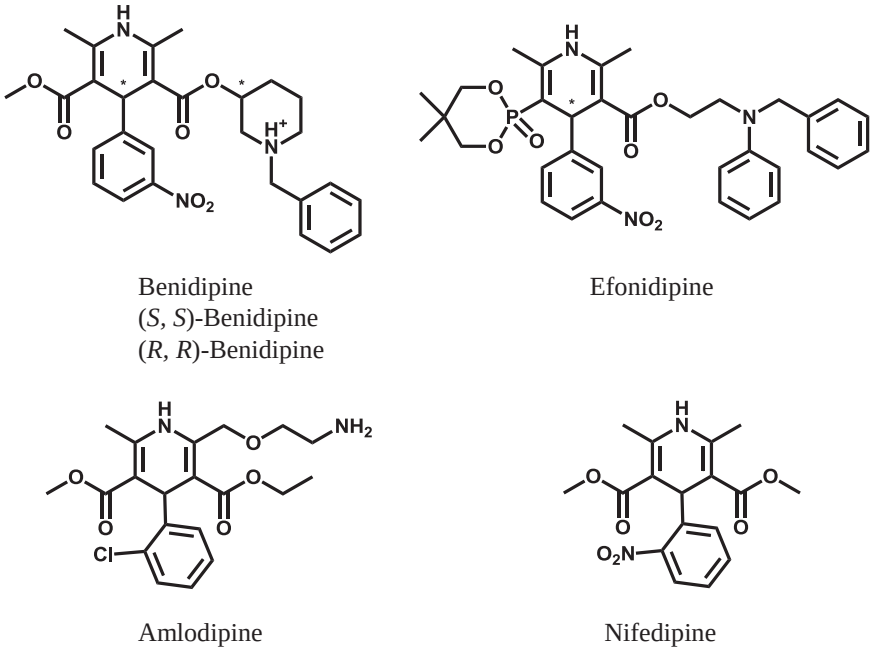

Fig. 1. Chemical structures of the dihydropyridine Ca^{2+} channel blockers used in this study.