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5*E*- and 5*Z*-farnesylacetones from *Sargassum siliquastrum* as novel selective L-type calcium channel blockers

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ABSTRACT

A specific blocker of L-type Ca^{2+} channels may be useful in decreasing arterial tone by reducing the open-state probability of L-type Ca^{2+} channels. The aim of the present study was to evaluate the farnesylacetones, which are major active constituents of *Sargassum siliquastrum*, regarding their vasodilatation efficacies, selectivities toward L-type Ca^{2+} channels, and in vivo antihypertensive activities. The application of 5E-(farnesylacetone 311) or 5Z-farnesylacetone (farnesylacetone 312) induced concentration-dependent vasodilatation effects on the basilar artery that was pre-contracted with depolarization and showed an ignorable potential role of endothelial-derived nitric oxide. We also tested farnesylacetone 311 or 312 to determine their pharmacological profiles for the blockade of native L-type Ca^{2+} channels in basilar arterial smooth muscle cells (BASMCs) and ventricular myocytes (VMCs), cloned L- ($\alpha1C/\beta2a/\alpha2\delta$), N- ($\alpha1B/\beta1b/\alpha2\delta$), and T-type Ca^{2+} channels ($\alpha1G$, $\alpha1H$, and $\alpha1I$). Farnesylacetone 311 or 312 showed greater selectivity toward the L-type Ca^{2+} channels among the tested voltage-gated Ca^{2+} channels. The ranked order of the potency for farnesylacetone 311 was cloned $\alpha1C$ L-type (BASMC) L-type (VMCs) > $\alpha1B$ > $\alpha1H$ > $\alpha1I$ > $\alpha1G$ and that for farnesylacetone 312 was cloned $\alpha1C$ L-type (BASMCs) L-type (VMCs) > $\alpha1H$ > $\alpha1G$ > $\alpha1B$ > $\alpha1I$. The oral administration of the farnesylacetone 311 (80 mg/kg) conferred potent, long-lasting antihypertensive activity in spontaneous hypertensive rats, but it did not alter the heart rate.

1. Introduction

Calcium influx via voltage-gated Ca^{2+} channels regulates neuronal excitability, neuronal growth and differentiation, and neurotransmitter release within the nervous system and triggers smooth muscle contraction in the vasculature and gastrointestinal tract (Doering and Zamponi, 2003). Among the voltage-gated Ca^{2+} channel subtypes, which are classified as high voltage-activated (L, N, P/Q, R) and low voltage-activated (T) channels, L-type Ca^{2+} channels are the primary pathway for the voltage-activated Ca^{2+} influx that triggers excitation–contraction coupling in vascular smooth muscle cells (Nelson et al., 1990). Functionally, L-type Ca^{2+} channels display a unitary conductance of 20 pS, and are highly sensitive to dihydropyridine (DHP), which constitutes a group of small organic compounds that are based on a core pyridine structure and include nifedipine, nimodipine, and isradipine (Bangalore et al., 1994; Sun and Triggle, 1995). Physiologically, L-type Ca^{2+} channels inactivate slowly during sustained depolarization, so that the Ca^{2+} influx is sufficient to mediate the pressure-induced constriction of resistance vessels and contribute to the dynamic autoregulation of the systemic and cerebral arteries (Nelson et al., 1990; Narayanan et al., 1994). Therefore, sustained Ca^{2+} influx via L-type Ca^{2+} channels maintains a tonic level of vascular contraction and provides an excitatory template for endogenously vasoactive substances. In hypertension, however, the development of anomalous arterial tone is associated with an increased expression of L-type Ca^{2+} channel $\alpha1C$ subunits that are upregulated by stretch-dependent depolarization and constriction, which is referred to as the myogenic response and plays a critical role in regulating blood pressure and flow (Nelson et al., 1990; Wang et al., 1999; Cox and

Abbreviations: BASMCs, basilar arterial smooth muscle cells; VMCs, ventricular myocytes.

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Rusch, 2002). If blood pressure is not restored during the progression of hypertension, the vascular smooth muscle arterial cells appear to electronically remodel themselves as an adaptive response that occur to sustain the higher Ca^{2+} influx required to support persistent arterial contraction (Sonkusare et al., 2006). These electrical remodeling processes are induced by a loss of resting membrane K^+ conductance, and they increase the number of functional L-type Ca^{2+} channels, which results in increased L-type α1C subunit expression (Harder et al., 1985; Wilde et al., 1994; Pesic et al., 2004). Therefore, to treat essential hypertension, organic Ca^{2+} channel blockers (CCBs) including DHP, phenylalkylamines, and bezothiazipines are used to reduce the open-state probability of the L-type Ca^{2+} channels (Narita et al., 1983; Godfraind et al., 1991).

Hypertension is a major key risk factor for endothelial dysfunction, obesity, renal dysfunction, diabetes, coronary arterial disease, and stroke (Ogihara et al., 2005). Therefore, the development of L-type Ca^{2+} channel-specific molecules with low toxicity profiles is urgently needed to decrease the side effects of antihypertensive drugs. Recently, a large group of marine-derived, structurally unique second metabolites that are useful to medicine has yielded a great number of drug candidates to treat various diseases (Mayer et al., 2007). While searching for vasoactive molecules from a marine natural library collected from the coast of the East Sea in Korea, we discovered that sargahydroquinoic acid from *Sargassum micracanthum* showed vasodilatation effects with a high potency on the cerebral and systemic arteries (Park et al., 2008a). Farnesylacetones from *Sargassum siliquastrum* conferred especially potent vasodilatation effects on the basilar and common carotid arteries (Park et al., 2008b). Therefore, the present study was designed to evaluate whether the inhibitory effects of farnesylacetone 311 or 312 on L-type Ca^{2+} channels were selective within the different subtypes of voltage-activated Ca^{2+} channels, including high voltage-activated (HVA) N-type Ca^{2+} channels and low voltage-activated (LVA) T-type Ca^{2+} channels, and to establish the antihypertensive activities of the farnesylacetones in vivo.

2. Materials & methods

2.1. Drugs

5*E*- (311) and 5*Z*-farnesylacetone (312) were isolated from the brown alga *S. siliquastrum* as described previously (Park et al., 2008a, b). Briefly, the brown alga *S. siliquastrum* was collected from the coast of the East Sea in Korea and stored in a deep freezer at −70 °C. The crude extract was obtained by methanol (MeOH) extraction and ethyl acetate partition. Finally, two pure farnesylacetones 311 and 312 were purified by C-18 reverse phase high performance liquid chromatography (HPLC) (YMC ODS-aqua-prep, 75% MeOH, 3 ml/min). Dulbecco's modified Eagle Medium (DMEM), HEPES-buffered saline (HBS), bovine serum albumin (BSA), penicillin, and streptomycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from GibcoBRL (Grand Island, Sarasota, FL, USA). Collagnase type A, protease type XIV, and papain were purchased from Roche Applied Science (Indianapolis, IN, USA). Other chemicals were of analytical grade.

2.2. Preparation of basilar artery and vascular smooth muscle cells from male white rabbits

Male New Zealand white rabbits weighing 2–2.5 kg were anesthetized by inhalating enflurane. To prepare of the smooth muscle cells, the basilar artery was rapidly isolated under sterile conditions and placed in a Ca^{2+}-free physiological salt solution (PSS) that contained the following components (in mM): 135 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5.5 glucose, and 23.8 $NaHCO_3$ (pH 7.4, adjusted with a 95% O_2 and 5% CO_2). Residual blood was rinsed from the lumen and adherent connective tissue, fat, and adventitia were carefully removed. The basilar artery was cut into pieces with fine scissors and incubated in Ca^{2+}-free PSS solution containing papain (2 mg/ml) and dithiothreitol (1.5 mg/ml) at 37 °C for 15 min. Thereafter, the pieces were resuspended and incubated in a Ca^{2+}-free PSS containing collagenase (2 mg/ml), dithiothreitol (1.5 mg/ml), and bovine serum albumin (1.5 mg/ml) at 37 °C for 15 min. The digested pieces were triturated with a blunted-tipped pipette until a sufficient number of single cells were isolated. The freshly isolated cells were used in electrophysiological experiments. For the measurement of tension, the basilar artery was cut into rings (3 mm) in a dissecting chamber filled with a Ca^{2+}-free PSS saturated with a 95% O_2 and 5% CO_2 mixture. The basilar ring was mounted using a pair of stainless steel hooks under a resting tension of 0.8 g in organ baths containing 15 ml of PSS, which was maintained 37 °C and bubbled with a 95% O_2 and 5% CO_2 mixture. One of the hooks was connected to a force displacement transducer (MLT050; ADInstruments, Colorado Springs, CO, USA) and the tension was recorded with chart 5 on a Powerlab/400 (ADInstruments). After an equilibration was performed for 30 min, each ring specimen was repeatedly exposed to the high K^+ solution (50 mM K^+), which was prepared by replacing NaCl with an equimolar concentration of KCl, until the responses became stable. Functional endothelial cells were confirmed by the ability of acetylcholine (1 μM) to induce relaxation.

2.3. Preparation of ventricular myocytes

Ventricular myocytes were enzymatically isolated from male Sprague-Dawley (SD) rats (200 g) as described previously. Briefly, the SD rats were anesthetized by an intra-peritoneal injection of pentobarbital sodium (50 mg/kg). The rat hearts were rapidly excised and perfused retrogradely with a Ca^{2+}-free Tyrode solution for 5 min through the aorta and then with Ca^{2+}-free Tyrode's solution containing collagenase type A (1 mg/ml) and protease type XIV (0.1 mg/ml) for 15 min. The ventricular myocytes were then perfused and dispersed with Ca^{2+} Tyrode's solution (200 μM Ca^{2+}) and stored at room temperature for 2 h before use. The Tyrode solution contained the following components (in mM): NaCl 137, KCl 5.4, HEPES 10, MgCl2 1, $CaCl_2$ 2, and glucose 10 (pH 7.4, adjusted with NaOH).

2.4. Cell culture and transfection of Ca^{2+} channels

Human Embryo Kidney (HEK 293) cells were obtained from the Korean Cell Line Bank (KCLB) and cultured in DMEM containing 10% FBS, penicillin (100 units/ml), and streptomycin (100 mg/ml) in a humidified CO_2 incubator (5% CO_2–95% air) at 37 °C. HEK 293 cells stably expressing T-type Ca^{2+} channel isoforms, α1G (GenBank accession number; GAN: AF027984), α1H (GAN: AF051946), and α1I (GAN: AF086827), were kindly provided by Dr. Edward Perez-Reyes (University of Virginia) and cultured in basal medium supplemented with 1 mg/ml G-418. To facilitate the heterologous expression of L-type Ca^{2+} channels, a calcium phosphate transfection kit (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's instructions. Briefly, before 24 h of transfection, HEK 293 cells were harvested and plated in a 35 mm culture dish containing 2×10^5 cells per dish. For the transient transfection of the L-type Ca^{2+} channels, a cDNA cocktail was prepared as follows: α1C 3 μg, β2a 1 μg, α2δ 1 μg, GFP 0.6 μg, 1 M $CaCl_2$ 7.5 μl, and ddH_2O to a final volume of 60 μl. The DNA cocktail was added to 60 μl of 2× HBS in an Eppendorf tube and stirred carefully. The mixture was incubated for 30 min at room temperature, during which time the precipitate formed. The precipitate was then added to HEK 293 cells in a 35 mm culture dish and incubated in a CO_2 incubator for 48 h.

2.5. Electrophysiology

Ca^{2+} channel currents were recorded using the whole-cell variant of the patch clamp technique (Hamil et al., 1981) as described previously (Ikeda, 1991; Jeong and Ikeda, 1998). The patch electrodes were