European Journal of Pharmacology 680 (2012) 22–27

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

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

Molecular and Cellular Pharmacology

Mechanism of osthole inhibition of vascular $Ca_v1.2$ current

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ARTICLE INFO

Article history:
Received 5 November 2011
Received in revised form 23 January 2012
Accepted 28 January 2012
Available online 4 February 2012

Keywords:
$Ca_v1.2$ channel current
Osthole
Rat tail artery myocyte
Traditional eastern medicine
Whole-cell patch-clamp

ABSTRACT

Osthole is a coumarin extracted from *Cnidium monnieri* (L.) Cusson. The medicinal plant is widely used in Vietnamese as well as Chinese traditional medicine as a vasodilating and antihypertensive agent. Here we have tested the proposition that the block of $Ca_v1.2$ channels is mainly responsible for its vascular activity. An in-depth analysis of the effect of osthole on $Ca_v1.2$ current ($I_{Ca1.2}$) was performed in rat tail artery myocytes using the whole-cell patch-clamp method. Osthole decreased $I_{Ca1.2}$ in a concentration- and voltage-dependent manner. At holding potentials of −50 and −80 mV, the pIC_{50} values were 4.78 ± 0.07 and 4.36 ± 0.08, respectively; the latter corresponded to the drug apparent dissociation constant for resting channels, K_R, of 47.8 μM. Osthole speeded up the inactivation kinetics of $I_{Ca1.2}$ and shifted the voltage dependence of the inactivation curve to more negative potentials in a concentration-dependent manner, with an apparent dissociation constant for inactivated channels (K_I) of 6.88 μM. Block of $I_{Ca1.2}$ was frequency-dependent and the rate of recovery from inactivation was slowed down. In conclusion, osthole is a vascular $Ca_v1.2$ channel antagonist stabilizing the channel in its inactivated state. This mechanism may account for the systolic blood pressure reduction induced by the drug in animal models of hypertension and points to osthole as a lead for the development of novel antihypertensive agents.

1. Introduction

Cnidium monnieri (L.) Cusson (Apiaceae) is a very small annual (or perennial) plant that is native to China and Vietnam, where it may be found growing on field edges, ditches and waste places. It also grows in Korea, Mongolia and Russia and has been introduced to the United States (Oregon) and Europe. The dried fruit of *Cnidium monnieri*, called "She chuang zi" in China and "Xà sàng tù " in Vietnam, has been used as a traditional remedy for male impotence, ringworm and blood stasis (Ou, 1989). Many compounds have been identified in *Cnidium monnieri*: coumarins such as osthole, imperatorin, bergapten, isopimpinellin, xanthotoxol, xanthotoxin, and biscoumarins such as cnidimonal and cnidimarin; glucosides; and sesquiterpenes (Cai et al., 2000; Zhang et al., 2007; Shin et al., 2011). Among them, osthole (7-methoxy-8-(3-methyl-2-butenyl)coumarin; Fig. 1) is the major component (its concentration in dried fruits is estimated to range between 4 and 22 mg/g (0.05–2.14%)) depending on the region of origin. Owing to its various pharmacological activities, osthole is considered to have many potential therapeutic applications (Hoult and Payá, 1996). It exhibits anti-inflammatory (Nakamura et al., 2009), anti-allergic (Matsuda et al., 2002) and neuroprotective effects (Chen et al., 2011; Mao et al., 2011). Recently it has achieved considerable attention owing to its broad spectrum of pharmacological effects, including anti-oxidant (Ji et al., 2010), estrogen-like (Hsieh et al., 2004), anabolic/anti-osteoporosis (Tang et al., 2010) and anti-tumor activities (Yang et al., 2010); hence, it is considered a scaffold for the development of novel chemotherapeutic agents (You et al., 2010).

Some *in vitro* pharmacological studies on either rat aorta (Ko et al., 1992) or on rabbit *corpus cavernosum* (Chen et al., 2000) have provided the evidence of osthole vasorelaxing activity. In fact, it was found to inhibit rat thoracic aorta contraction evoked by 80 mM K^+ thus suggesting a block of $Ca_v1.2$ channels. However, to our knowledge, the only report of its inhibition of L-type Ca^{2+} current refers to NG108-15 neuronal cells (Wu et al., 2002). Additionally, the *in vivo* osthole antihypertensive activity has been recently provided (Ogawa et al., 2007). Therefore, the aim of the present study was to assess the role played by vascular $Ca_v1.2$ channels in osthole-induced vasorelaxing activity. The results here presented demonstrate that osthole is a vascular $Ca_v1.2$ channel blocker and suggest that these channels are a crucial target for its vasorelaxing activity.

2. Materials and methods

2.1. Animals

All animal care and experimental procedures complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Animal Care and Ethics Committee

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doi:10.1016/j.ejphar.2012.01.038

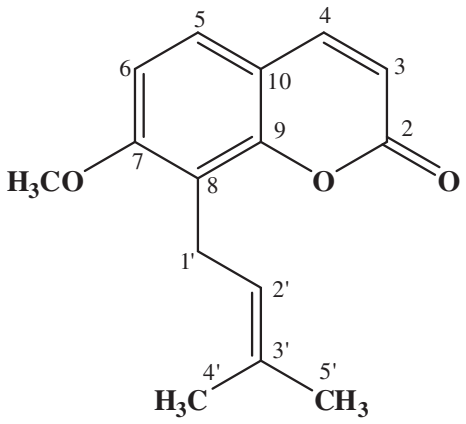

Fig. 1. Molecular structure of osthole (7-methoxy-8-(3-methyl-2-butenyl)coumarin).

of the Università degli Studi di Siena, Italy (27-1-2009). Male Wistar rats (300–400 g, Charles River Italia, Calco, Italy) were anesthetized (i.p.) with a mixture of Ketavet® (30 mg/kg ketamine; Intervet, Aprilia, Italy) and Xilor® (8 mg/kg xylazine; Bio 98, San Lazzaro, Italy), decapitated and exsanguinated. The tail was removed immediately, cleaned of skin and placed in external solution containing 20 mM taurine and 0.1 mM Ca^{2+} (see below for composition). The main tail artery was dissected free of its connective tissue.

2.2. Cell isolation procedure

Smooth muscle cells were freshly isolated from the tail main artery incubated at 37 °C in 2 ml of external solution containing 20 mM taurine and 0.1 mM Ca^{2+} (see below) containing 1.35 mg/ml collagenase (type XI), 1 mg/ml soybean trypsin inhibitor, and 1 mg/ml BSA, gently bubbled with a 95% O_2–5% CO_2 gas mixture, as previously described (Fusi et al., 2001). Cells exhibited an ellipsoid form (10–15 μm in width, 35–55 μm in length) and were continuously superfused with external solution containing 0.1 mM Ca^{2+} using a peristaltic pump (LKB 2132, Bromma, Sweden), at a flow rate of 400 μl/min.

2.3. Whole-cell patch clamp recordings

The conventional whole-cell patch-clamp method (Hamill et al., 1981) was employed to voltage-clamp smooth muscle cells. Recording electrodes were pulled from borosilicate glass capillaries (WPI, Berlin, Germany) and fire-polished to obtain a pipette resistance of 2–5 MΩ when filled with internal solution (see below). A low-noise, high-performance Axopatch 200B patch-clamp amplifier (Molecular Devices Corporation, Sunnyvale, CA, USA) driven by a personal computer in conjunction with an A/D, D/A board (DigiData 1200 A/B series interface, Molecular Devices Corporation) was used to generate and apply voltage pulses to the clamped cells and record the corresponding membrane currents. At the beginning of each experiment, the junction potential between the pipette and bath solution was electronically adjusted to zero. Cell break-in was accomplished by gentle suction at a holding potential (V_h) of −50 mV. Then, V_h was set either to −50 mV or to −80 mV. Micropipette seals had to be GΩ in nature with leak currents less than 0.25 pA/mV. Current signals, after compensation for whole-cell capacitance and series resistance (between 70 and 80%), were low-pass filtered at 1 kHz and digitized at 3 kHz prior to being stored on the computer hard disk. Electrophysiological responses were tested at room temperature (20–22 °C) only in those cells that were phase dense.

2.4. $I_{Ca1.2}$ recordings

$I_{Ca1.2}$ was always recorded in external solution containing 30 mM tetraethylammonium as well as 5 mM Ca^{2+}. Current was elicited with 250-ms clamp pulses (0.067 Hz) to 10 mV from a V_h of −50 mV or −80 mV. Cells clamped at a V_h of −80 mV expressed $Ca_v1.2$ but not $Ca_v3.1$ channels (see Petkov et al., 2001). Data were collected once the current amplitude had been stabilized (usually 7–10 min after the whole-cell configuration had been obtained). At this point, the various protocols were performed as detailed below.

Steady-state inactivation curves were obtained using a double-pulse protocol. Once various levels of the conditioning potential had been applied for 5 s, followed by a short (5 ms) return to the V_h, a test pulse (250 ms) to 10 mV was delivered to evoke the current. The delay between the conditioning potential and the test pulse allowed the full or near-complete deactivation of the channels simultaneously avoiding partial recovery from inactivation.

Activation curves were derived from the current–voltage relationships shown in Fig. 3. Conductance (G) was calculated from the equation $G = I_{Ca1.2}/(E_m - E_{rev})$, where: $I_{Ca1.2}$ is the peak current elicited by depolarizing test pulses in the range of −50 to 20 mV from V_h of −50 mV; E_m is the membrane potential; and E_{rev} is the reversal potential (181 mV, as estimated with the Nerst equation). G_{max} is the maximal Ca^{2+} conductance (calculated at potentials ≥5 mV). The ratio G/G_{max} was plotted against the membrane potential and fitted with the Boltzmann equation.

A two-pulse protocol was applied to measure the time course of recovery from inactivation: 2-sec clamp pulses to 10 mV from a V_h of −50 mV were followed by a return to the V_h of variable duration to allow some channels to recover from inactivation. A second pulse (250 ms) to 10 mV was delivered to determine how much recovery had occurred during the time interval.

K^+ currents were blocked with 30 mM tetraethylammonium in the external solution and Cs^+ in the internal solution (see below).

Current values were corrected for leakage using 10 μM nifedipine, which completely blocked $I_{Ca1.2}$.

2.5. Solutions for $I_{Ca1.2}$ recordings

The external solution contained (in mM): 130 NaCl, 5.6 KCl, 10 HEPES, 20 glucose, 1.2 $MgCl_2 \cdot 6\ H_2O$, and 5 Na-pyruvate (pH 7.4). For cell isolation, external solution containing 20 mM taurine was prepared by replacing NaCl with equimolar taurine. $CaCl_2$ (5 mM, final concentration) and tetraethylammonium (30 mM) were added to the external solution for $I_{Ca1.2}$ recordings. The internal solution (pCa 8.4) consisted of (in mM): 100 CsCl, 10 HEPES, 11 EGTA, 2 $MgCl_2 \cdot 6\ H_2O$, 1 $CaCl_2$, 5 Na-pyruvate, 5 succinic acid, 5 oxalacetic acid, 3 Na_2-ATP, and 5 phosphocreatine; pH was adjusted to 7.4 with CsOH. The osmolarity of the 30 mM tetraethylammonium- and 5 mM Ca^{2+}-containing external solution (320 mosmol) and that of the internal solution (290 mosmol; Stansfeld and Mathie, 1993) were measured with an osmometer (Osmostat OM 6020, Menarini Diagnostics, Florence, Italy).

2.6. Materials

The materials used included: collagenase (type XI), trypsin inhibitor, BSA, tetraethylammonium chloride, EGTA, taurine and $CdCl_2$ (from Sigma Chimica, Milan, Italy). Osthole was isolated from *Cnidium monnieri* (L.) Cusson fruits harvested in Vietnam, using the procedure described by Ty et al. (1998). In brief, the dried fruits were extracted with *n*-hexane/acetone (90:10) for three times under reflux for 4 h. The solvent was removed *in vacuo* to yield a yellow solid extract, which was then dissolved in acetone. Osthole was crystallized at 4–10 °C and filtered off to obtain osthole 98–99% pure as white needles. Osthole purity was evaluated by HPLC-MS methods,