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Research paper

Relaxant effect of flavonoid naringenin on contractile activity of rat colonic smooth muscle

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ABSTRACT

Ethnopharmacological relevance: Disturbed gastrointestinal (GI) motility can be associated with smooth muscle abnormalities and dysfunction. Exploring innovative approaches that can modulate the disturbed colonic motility are of great importance for clinical therapeutics. Naringenin, a flavonoid presented in many traditional Chinese herbal medicines, has been shown to have a relaxant effect on different smooth muscles. The aim of the present study was to investigate the effect of naringenin on regulation of GI motility.

Material and methods: Mechanical recording was used to investigate the effect of naringenin on isolated rat colonic smooth muscle spontaneous contractions. Whole cell patch clamp, intracellular $[Ca^{2+}]$ concentration ($[Ca^{2+}]_i$) and membrane potential measurements were examined on primary cultures of colonic smooth muscle cells (SMCs). A neostigmine-stimulated rat model was utilized to investigate the effect of naringenin *in vivo*.

Results: Naringenin induced a concentration-dependent inhibition (1–1000 μM) on rat colonic spontaneous contraction, which was reversible after wash out. The external Ca^{2+} influx induced contraction and $[Ca^{2+}]_i$ increase were inhibited by naringenin (100 μM). In rat colonic SMCs, naringenin-induced membrane potential hyperpolarization was sensitive to TEA and selective large-conductance calcium-activated K^+ (BK_{Ca}) channel inhibitor iberiotoxin. Under whole cell patch-clamp condition, naringenin stimulated an iberiotoxin-sensitive BK_{Ca} current, which was insensitive to changes in the $[Ca^{2+}]_i$ concentration. Furthermore, naringenin significantly suppressed neostigmine-enhanced rat colon transit *in vivo*.

Conclusion: Our results for the first time demonstrated the relaxant effect of flavonoid naringenin on colon smooth muscle both *in vitro* and *in vivo*. The relaxant effect of naringenin was attributed to direct activation of BK_{Ca} channels, which subsequently hyperpolarized the colonic SMCs and decreased Ca^{2+} influx through VDCC. Naringenin might be of therapeutic value in the treatment of GI motility disorders.

1. Introduction

Large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels are broadly expressed in the GI smooth muscle cells (SMCs) (Benham et al., 1985; Carl and Sanders, 1989). BK_{Ca} channels, regulated by voltage and $[Ca^{2+}]_i$, play a key role in controlling cell excitability of SMCs (McManus, 1991; Lawson, 1996). Blockade of BK_{Ca} with charybdotoxin increased ileum spontaneous activity (Hong et al., 1997). Conversely, BK_{Ca} activation reduced contractility of ileum smooth muscle (Dela Pena et al., 2009), so BK activators may be reasonable options for spasmodic motility disorders. Several synthetic and natural compounds that activate BK_{Ca} channels, referred to as BK openers, have been reported (Hu et al., 1995; Dela Pena et al., 2009). However, no BK_{Ca} channel modulator has been approved for clinical use so far.

Naringenin, one of the most commonly consumed flavonoid compounds within our regular diet (Erlund, 2004), has been reported to have relaxing effects on contractility of vascular smooth muscle (Herrera et al., 1996; Ajay et al., 2003). Activation of BK_{Ca} channel has been proposed to explain, at least in part, the vasodilatory activity of naringenin (Calderone et al., 2004; Saponara et al., 2006). However, little attention has been attributed to naringenin-induced relaxation of GI smooth muscle. Studies have shown that *Zhishi* decoction, in which naringenin presents considerable amount (Liu, 2008), could inhibit rat and

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rabbit colon smooth muscle contraction (Zhang et al., 2006; Liu et al., 2010). Naringenin, per se, has been reported to reduce gastric tone (Hammad and Abdalla, 1997) and intestinal peristalsis (Gharzouli and Holzer, 2004). However, the effect of naringenin on colonic smooth muscle cells as BK modulators is not yet known.

In the present study, we investigated the effect of naringenin on isolated rat colonic smooth muscle spontaneous contraction and *in vivo* intestine motility, and examined the possible underlying cellular mechanisms.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats of approximately 200 g body weight were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou). Rats, kept on a 12:12 light/dark cycle (light from 08:00 to 20:00), were group-housed (five per cage) in a room maintained at 24 ± 0.5 °C and with free access to food and water. Rats were sacrificed by CO_2 inhalation and distal colon tissues were dissected out. The ethics committee of Sun Yet-sen University approved all procedures.

2.2. Materials

Medium 199, fetal bovine serum (FBS), penicillin–streptomycin, Hanks' balanced salt solution (HBSS), sodium pyruvate and trypsin were purchased from Life Technologies (Gibco®, New York). Naringenin, Collagenase II, L-NG-nitroarginine (L-NNA), paraformaldehyde (PFA), tetrodotoxin (TTX), sodium dodecyl sulfate (SDS), tetraethylammonium chloride (TEA), 2-Aminoethyl diphenylborinate (2-APB), EGTA, ATP, iberiotoxin, nifedipine, neostigmine were from Sigma-Aldrich (St Louis, MO). Bovine serum albumin (BSA) was from Roche Diagnostics (Indianapolis, IN), Fluo-3/AM and bis (1,3-dibutylbarbibturic acid) trimethine oxonol ($DiBAC_{4(3)}$) were from Molecular Probes (Eugene, OR). Primary antibody (rat monoclonal anti-alpha smooth muscle actin) and FITC-labeled secondary antibody were from Boster (Wuhan, China).

Naringenin applied in all the experiments was (±)-Naringenin. Drugs were dissolved in 1% DMSO. Control colonic strips subjected to 1% DMSO did not exhibit significant changes in the force and frequency of contractions over the observation time for more than 2 h.

2.3. Tissue preparations and mechanical activity recording

Colon segments were split open longitudinally and the entire epithelium was gently peeled off using fine forceps. The segments (~1 cm long) in the direction of longitudinal muscle fibers were bathed in 10 ml Krebs–Henseleit (K–H) solution (37 °C) with the lower end of each strip anchored to a J-hook. Isometric tension was recorded by a force transducer attached to a cotton thread ligated to the upper end of muscle strips. Mechanical activity was digitized by a signal collection and analysis system (BL-420E, Chengdu Technology & Market Co. Ltd., China) as described elsewhere (Fang et al., 2005). The strips were subjected to an initial tension of 1 g and were allowed to equilibrate for at least 60 min before starting the experiment. The K–H solution contained (in mM) 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11.1 D-glucose.

2.4. Primary cultures of SMCs

Distal colon (~5 cm long) was dissected out and fecal contents were washed away using a 50 ml syringe filled with cold PBS. The epithelium layer was removed from the smooth muscle layer using dissection scissors under a stereo microscope (Leica). The smooth muscle layers along with the *myenteric plexus* were minced and digested for 30–40 min at 37 °C in an enzyme solution contained (in mg/ml) 1 collagenase II, 2 BSA, 0.4 ATP. The entire cell suspension was passed through a sieve (100-mesh). Disaggregated cells were suspended in M199 supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were seeded onto glass coverslips placed in a culture plate. The cells were maintained in culture at 37 °C in a humidified 5% CO_2 air atmosphere.

2.5. Immunostaining

SMCs grown to 60–70% confluence were washed 3 times with PBS and then fixed in 4% PFA for 30 min. After removing the PFA by washing 3 times with glycine solution, cells were permeablized with 1% SDS for 5 min and then placed in blocking solution (1% BSA in PBS) for 30 min. Cells were incubated at room temperature with a primary antibody for 2 h. Subsequently, cells were washed in PBS and incubated with a FITC-labeled secondary antibody for another 1 h. Cells were visualized under a fluorescence microscope (Nikon, Tokyo, Japan).

2.6. Measurement of $[Ca^{2+}]_i$

SMCs were seeded on 25 mm round glass coverslips and grown at 37 °C until reaching 70–80% confluence. Cells were washed in normal physiological saline solution (NPSS; 137 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM HEPES, and 11 mM glucose), and incubated with 10 μM Fluo-3/AM in NPSS for 1 h at room temperature. The coverslips were then transferred to a 2-ml chamber perfused with NPSS. Fluorescence signal was monitored and simultaneously recorded by a Laser Scanning Confocal Imaging System (TCS SP2; Leica Microsystems, Germany). Ca^{2+}-free physiological saline solution (Ca^{2+}-free PSS) was prepared by omitting Ca^{2+} and adding 2 mM EGTA to the NPSS. Change of fluorescence intensity after drug treatments was normalized with the initial intensity.

2.7. Whole cell patch-clamping recordings

SMCs were seeded on 14 mm round glass coverslips and grown at 37 °C until reaching 50–60% confluence. Cells were transferred to a 2-ml chamber which was placed on the stage of an upright microscope system (BX51WI; Olympus, Japan). Data were acquired by using a multiclamp 700 A amplifier and Digidata 1322 interface (AXON Instruments, Foster City, CA). Signals were filtered at 10 kHz and sampled at 100 kHz. Patch pipettes were pulled from glass pipettes using P-97 horizontal puller (Sutter Instrument Co., Novato, CA). Pipette resistance was between 3 and 7 MΩ and the seal resistance was achieved above 5 GΩ.

The internal pipette solution contained (in mM) 140 KCl, 1 $MgCl_2$, 1 EGTA, 0.55 $CaCl_2$, 10 HEPES (pH 7.2); free $[Ca^{2+}]$ in the pipette solution is 50 nM. In some experiments, $CaCl_2$ was increased to 1.09 and 1.19 mM in order to get free $[Ca^{2+}]_i$ of 100 and 200 nM, respectively. The external bath solution contained (in mM): 135 NaCl, 4 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, and 5 glucose (pH 7.4). After obtaining the whole-cell configuration, the membrane potential was clamped at −40 mV. Voltage-step-induced whole cell BK currents were elicited by step depolarization between −20 mV and +100 mV in increments of 20 mV with

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