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Antispasmodic effect of selected *Citrus* flavonoids on rat isolated jejunum specimens

Marta Mendel*, Magdalena Chłopecka, Natalia Dziekan, Wojciech Karlik

Warsaw University of Life Sciences, Faculty of Veterinary Medicine, Department of Preclinical Sciences, Division of Pharmacology and Toxicology, 8, Ciszewskiego St., 02-786 Warsaw, Poland

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apamin (PubChem CID: 57043712)
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diosmin (PubChem CID: 5281613)
glibenclamide (PubChem CID: 3488)
hesperetin (PubChem CID: 72281)
hesperidin (PubChem CID: 10621)
indomethacin (PubChem CID: 3715)
N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) (PubChem CID: 39836)
tetraethylammonium (PubChem CID: 5413)

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ABSTRACT

Citrus flavonoids are acknowledged for numerous pharmacological activities, including the myorelaxant effect on various smooth muscles. However, there is no data on their effect on jejunum contractility. Therefore, the aim of the study at hand was to evaluate the impact of hesperetin and diosmetin along with their glycosides on the motoric activity of intestine and to verify the possible mechanism of hesperetin-induced effect. The experiments were performed on rat isolated jejunum strips and were conducted under isometric conditions.

Hesperetin and diosmetin, but not hesperidin and diosmin, dose-dependently (10–100 μM) and reversibly inhibited acetylcholine (1 μM) and KCl (80 mM) induced contractile activity. The antispasmodic effect of hesperetin was partially blocked by 4-aminopyridine (100 μM), glibenclamide (100 μM) and NG-nitro-L-arginine methyl ester (L-NAME, 100 μM). By contrast, apamin (0.1 μM), tetraethylammonium (500 μM) and methylene blue (10 μM) did not affect the magnitude of hesperetin-induced myorelaxant effect. Indomethacin (10 μM) increased the force of hesperetin-evoked reaction.

In conclusion, hesperetin and diosmetin are potent myorelaxant agents. The antispasmodic effect of hesperetin is partially mediated by fast current low-voltage activated K^+ channels, voltage-independent K+ channels and involves the nitric oxide pathway. Finally, hesperetin shows a synergistic effect with indomethacin towards jejunal KCl-precontracted smooth muscle.

1. Introduction

Citrus flavonoids belong to the group of aromatic secondary plant metabolites which have been acknowledged for their potent antioxidant and anti-inflammatory effects (Parhiz et al., 2015). They are also known to prevent cardiovascular disorders, like coronary heart disease (Gross, 2004) and show anticancer and chemopreventive properties towards e.g. lung and colon tumors (Roohbakhsh et al., 2015) as well as to support treatment of numerous chronic diseases like e.g. asthma (Seyedrezazadeh et al., 2015). *Citrus* fruits and juices are prominent sources of dietary phenolic compounds, containing mostly flavanones and flavones (Gattuso et al., 2007), e.g. hesperetin and diosmetin with corresponding glycosyl derivatives, i.e. hesperidin (hesperetin 7-O-rutinoside) and diosmin (diosmetin 7-O-rutinoside). According to Gattuso et al. (2007), the concentration of hesperidin and diosmin in *Citrus* fruits juices might be as high as 86.1 (*Citrus clementina*) and 7.2 (sweet orange; unspecified commercial orange juices samples) mg/100 mL, respectively. Hesperidin was found to be the most abundant flavonoid and widely distributed in over 60 *Citrus* samples (Kawaii et al., 1999). Besides dietary intake of the *Citrus* flavonoids, people might be exposed to these phytocompounds through diet supplements and various herbal medicines. Hesperidin along with diosmin are commonly used as combination products that proved to be beneficial for human with chronic venous insufficiency (Garg et al., 2001), haemorrhoids (Cospite, 1994) and for the prevention of postoperational thromboembolism (Tsimoyiannis et al., 1996).

Hesperidin and other hesperetin glycosides were found to be converted to aglycone, hesperetin, and further to phenolic acids in the intestine (Kim et al., 1998). However, the aglycone form was detected in urine and plasma (Ameer et al., 1996) which indicates that at least partially hesperetin is absorbed from the gut before being metabolized by bacteria. There are also promising results of attempts of

* Corresponding author.
E-mail address: marta_mendel@sggw.pl (M. Mendel).

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using hesperetin in modified formulations in order to increase its bioavailability (Takumi et al., 2012). Moreover, it was shown that the enzymes of bacteria of the intestinal microflora are responsible for the metabolic reduction of diosmetin to its flavanone analogue hesperetin through the reduction of the 2,3 double bond of the C-ring (Spanakis et al., 2009).

Among pharmacological activities assigned to flavonoids there are well documented vasodilatory, spasmolytic and antidiarrheal effects (Garg et al., 2001; Romano et al., 2013) that clearly indicate the impact of numerous flavonoids on smooth muscle. However, to the best of our knowledge, there is no data on hesperetin, diosmetin and their glycosides effect on jejunal contractility although the altered gastrointestinal motility is mentioned as one of the mechanisms underlying several problems in human, like irritable bowel syndrome or leaky gut syndrome (Kiefer and Ali-Akbarian, 2004, Canavan et al., 2014). The global prevalence of irritable bowel syndrome is equivalent to 11.2% (Lovell and Ford, 2012) but in some communities, e.g., USA, IK, Greece, Iceland, it may exceed 20% (Canavan et al., 2014). Therefore, the aim of the study at hand was to evaluate the impact of selected *Citrus* flavonoids on the motoric activity of isolated jejunum specimens and investigate the mechanism of hesperetin-induced antispasmodic effect.

2. Materials and methods

2.1. Chemicals and incubation media

Acetylcholine chloride (ACh), 4-aminopyridine (4-AP), apamin, dimethylsulfondioxide (DMSO), diosmetin, diosmin, hesperetin, hesperidin, glibenclamide, N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), methylene blue and tetraethylammonium (TEA) (Sigma Chemicals Co, St. Louis, USA), $CaCl_2$, (Merck, Darmstadt, Germany), NaH_2PO_4 (Fluka Chemie, AG, Buchs, Switzerland), all other salts needed for the preparation of the incubation media: NaCl, KCl, $MgSO_4$, $NaHCO_3$ and glucose (Avantor Performance Materials, Gliwice, Poland) were used for preparing and performing the experiments. Stock solutions of hesperetin and diosmetin were prepared in DMSO and serial dilutions were made in deionized water. Apamin was dissolved in deionized water, glibenclamide and indomethacin in 0.5% DMSO. All other reagent agents were dissolved in the incubation medium. Modified Krebs-Henseleit solution (M K-HS) containing: NaCl (123.76 mM), KCl (5 mM), $CaCl_2$ (2.5 mM), $MgSO_4$ (1.156 mM), $NaHCO_3$ (14.5 mM), KH_2PO_4 (2.75 mM) and glucose (12.5 mM), was employed as an incubation medium for jejunum strips and was maintained at pH 7.35–7.45 throughout the long-term experiments, while being heated to 37 °C and bubbled with carbogen (95% O_2+5% CO_2). In some experiments the specimens were contracted by K^+-rich M K-HS (80 mM KCl).

2.2. Collection and preparation of rat isolated jejunum strips

Male Wistar rats (250 g) were kept under standard laboratory conditions with free access to drinking water and a pallet of food (Sniff Special Diäten, GmbH, Germany). The study was approved by the local ethics committee (approval number 8/2011). The rats were euthanized by asphyxiation with CO_2. Jejunum was removed, washed, released from mesenteric attachment and cut into smaller segments of approx. 15–20 mm in length. Whole thickness segments were suspended in the direction of longitudinal smooth muscle fibers in organ baths of 5 mL each, filled with modified Krebs-Henseleit solution and continuously gassed with carbogen (95% O_2 and 5% CO_2). The tissues were clamped distally to hooks and proximally attached to the isometric force transducers (F30, type 372, Hugo Sachs Elektronik, Harvard Apparatus, March, Germany). All experiments were carried out under a load of 0.01 N. The isometric force transducer was linked to an analogue-digital registration set (PowerLab, ADInstruments, Bella Vista, Australia) by a bridge amplifier (DBA, type 660, Hugo Sachs Elektronik, Harvard Apparatus, March, Germany). Recordings of the strips' motoric activity were then registered by LabChart v7.0 program. All the calculations and analysis were completed by LabChart Reader v8.1.1 program and Excel (MS Office XP Professional).

2.3. Design of experiments

Jejunum segments were allowed to equilibrate for 60 min with rinsing every 15 min before starting the experiment. Then, the contractility of specimens was verified by the multiple application of acetylcholine (1 μM) and KCl (80 mM). Only jejunum strips which exhibited clear spontaneous contractility and in which acetylcholine and KCl responses were reproducible and stabilized were used for the proper part of the study. The further course of experiments was dependent on the aim of the study and included: (*i*) jejunum strips treatment with acetylcholine dissolved in DMSO (0.5%) in order to verify their response to the solvent (control reaction) followed by the application of one of the tested flavonoids, i.e. hesperetin, hesperidin, diosmetin or diosmin, in a non-cumulative manner, in a concentration range of 0.001–100 μM on acetylcholine-precontracted specimens; alternatively the administration of acetylcholine was preceded by a 10-min preincubation of jejunum preparations in the presence of one of the selected flavonoids; (*ii*) the application of hesperetin, hesperidin, diosmetin or diosmin in a concentration of 100 μM on jejunum strips precontracted with KCl (80 mM); flavonoids were added as soon as the tonic component of KCl-induced response started, cumulative application of tested agents was avoided, since KCl-induced contraction progressively declined after about 5 min; (*iii*) 10-min preincubation of jejunum specimens in the presence of one of the pharmacological antagonists (4-aminopyridine [100 μM], apamin [0.1 μM], glibenclamide [100 μM], indomethacin [10 μM], N_ω-Nitro-L-arginine methyl ester hydrochloride [100 μM], methylene blue [10 μM] and tetraethylammonium [500 μM]) followed by acetylcholine or KCl administration and hesperetin (100 μM) addition as soon as the tonic component of acetylcholine or KCl-induced response started. In the control trial the 10-min preincubation with pharmacological antagonists followed by the administration of acetylcholine or KCl did not involve hesperetin. The preincubation in the presence of 4-aminopyridine, apamin, glibenclamide or tetraethylammonium was followed by acetylcholine application whereas the preincubation involving indomethacin, N_ω-Nitro-L-arginine methyl ester hydrochloride or methylene blue was followed by KCl administration. Each jejunum specimen was used for only one concentration-response curve or for testing the effect of only one blocker on hesperetin-induced relaxation of acetylcholine or KCl-precontracted strips. Each flavonoid and each pharmacological blocker were tested on six segments of rat jejunum collected from six different animals. At the end of each set of experiments the preparations were re-exposed to the reference substance, acetylcholine (1 μM), in order to verify their reactivity.

2.4. Expression of the obtained results and statistical analysis

Registration of smooth muscle activity expressed as changes in the strips' tension was performed throughout the whole experiment. The effect of all the examined substances was based on changes in the smooth muscle strips' and calculated as AUC (area under the curve). The results aimed at studying the dose-effect relationship for all four flavonoids are expressed as percentage of the reaction caused either by acetylcholine in the optimal dose (1 μM) or KCl (80 mM) applied at the beginning of each experiment. The contraction provoked by acetylcholine or KCl in the reference dose was defined as 100% (control). The results conducted to explain the mechanism of hesperetin-provoked antispasmodic effect are expressed as percentage of acetylcholine (1 μM) or KCl (80 mM) induced contraction in the presence of the studied pharmacological blocker. The response of jejunum strips to