Steroids 79 (2014) 64–69

Contents lists available at ScienceDirect

Steroids

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

The flavonoid chrysin, an endocrine disrupter, relaxes cholecystokinin- and KCl-induced tension in male guinea pig gallbladder strips through multiple signaling pathways

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ARTICLE INFO

Article history:
Received 30 April 2013
Received in revised form 28 October 2013
Accepted 9 November 2013
Available online 26 November 2013

Keywords:
Chrysin
Gallbladder
Phytoestrogen
Calcium
Smooth muscle
Relaxation

ABSTRACT

The bioflavonoids have effects on vascular smooth muscle and gastrointestinal smooth muscle. The flavone and phytoestrogen, chrysin, has been shown to have a vasorelaxant effect on resistance blood vessels. This effect was mediated by nitric oxide (NO). Chrysin inhibited aromatase/estrogen biosynthesis in postmenopausal women. The purpose of this study was to determine if chrysin had an effect on cholecystokinin- or KCl-induced tension in male guinea pig gallbladder strips. In addition, the second messenger(s) system(s) that mediated the effect were to be determined. A pharmacologic approach was used. Male guinea pig gallbladder strips were placed in *in vitro* chambers filled with Krebs solution, maintained at 37 °C, and gassed with 95% O_2–5% CO_2. Changes in tension were recorded using a polygraph.

It was shown that the PKA/cAMP second messenger system mediated part of the observed chrysin-induced relaxation of cholecystokinin-induced tension, the PKC system also mediated part of the relaxation, and the inhibition of both extracellular Ca^{2+} entry and intracellular Ca^{2+} release also mediated the chrysin-induced relaxation. This is the first report of chrysin having an effect on gallbladder smooth muscle contraction.

1. Introduction

The flavonoids comprise a large group of polyphenolic compounds. The flavone chrysin (5,7-dihydroxyflavone) is present in honey and propolis and, in low concentrations, in fruits, vegetables, and beverages [1,2]. The flavonoids exhibit biologic effects including reducing plasma levels of low-density lipoproteins, inhibiting platelet aggregation, and reducing cell proliferation [3–5]. The flavonoids also have pharmacologic actions on vascular and intestinal smooth muscle tone [6–11]. Many of the flavonoids have chemical structures similar to estrogen and can act as endocrine disrupting agents [12]. Chrysin has vasodilator effects on resistance blood vessels, which depend partially on a functional epithelium and appear to also rely on the NO/cGMP pathway [13]. The chrysin-induced stimulated NO release was shown to be calcium independent and possibly mediated by PI3-kinase [14].

Chrysin was shown to produce a concentration-dependent relaxation of the contractile response induced by noradrenalin, KCl, or phorbol 12-myristate-13 acetate in rat aortic rings. Several mechanisms were suggested to mediate the vasodilatory effects of flavonoids including inhibition of PKC, inhibition of cyclic nucleotide phosphodiesterases, and/or decreased Ca^{2+} uptake [7]. Chrysin as a phytoestrogen may function as a chemoprotective agent by inhibiting aromatase/estrogen biosynthesis in post menopausal women [15]. Chrysin, as an aromatase inhibitor, has also been shown to produce DNA breaks and/or point mutation in HepG2 cells [15]. It also inhibited cell growth due to the induction of apoptosis [16]. Chrysin also caused inhibition of tonic and phasic contractions of the rat isolated mesenteric vascular bed. PDE activation was suggested to be the mediator of the observed inhibition of isolated vascular bed activity [12]. In the rat isolated thoracic aorta chrysin and other flavones caused a concentration-dependent inhibition of the contractile responses to exogenous applications of Ca^{2+} and the release of intracellular Ca^{2+} stimulated by phenylephrine [11]. Chrysin relaxed the contractions induced by noradrenaline in isolated endothelium-intact rat aortic rings. Endothelium removal or L-NAME inhibited this relaxant effect; thus, the chrysin-induced vasorelaxation is NO-dependent [16,17]. Ajay et al. [18] also showed that NO release and inhibition of Ca^{2+} influx and intracellular Ca^{2+} release mediated the chrysin-

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http://dx.doi.org/10.1016/j.steroids.2013.11.012

induced relaxant effect in the isolated rat thoracic aorta. Villar et al. [13] using the isolated rat mesenteric vascular bed also showed that the chrysin-induced relaxation of the vascular bed was mediated by the NO/cGMP pathway. Villar et al. [14] using the rat isolated aorta proposed that the chrysin-induced relaxation was mediated by chrysin-stimulated NO release that was Ca^{2+} independent and possibly mediated by PI3-kinase.

The purpose of this study was to determine if chrysin had a relaxant effect on either cholecystokinin octapeptide (CCK) or KCl-induced tension in male guinea pig gallbladder strips and determine which second messenger(s) mediated the relaxant effect.

2. Experimental

The experiments were performed under a protocol (#275) approved (April 1, 2012) by the Animal Care Committee-Health Sciences of the University of Alberta. Male Hartley guinea pigs (200–375 g body weight) were killed by decapitation. The gallbladder was removed, cleaned, and placed in Krebs-Henseleit solution (KHS) that was gassed with 95% O_2 and 5% CO_2. The composition of the KHS was (mM) NaCl, 115; KCl, 5; $CaCl_2$, 2.1; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; and glucose, 11. Each gallbladder was cut into strips (1.5 × 0.5 cm) and maintained in Sawyer-Bartlestone chambers filled with KHS, maintained at 37 °C, and gassed with 95% O_2 and 5% CO_2. An optimum resting tension of 0.7 g was determined previously and used in the study [19,20].

The force developed by the gallbladder strips was measured with Grass FT03 force displacement transducers and recorded on a Grass 7D polygraph (Grass Instruments Co., Quincy MA). Isolated strips were equilibrated in the chambers for 45 min prior to determining their suitability for use. Each chamber had 2 μM (final concentration) atropine added, in every experiment, 3 min prior to 1.0 nM cholecystokinin octapeptide (CCK). The tension was measured. This was followed by 3 changes of KHS. The test was repeated twice with 25 min between tests. A repeatable minimum active tension of 0.5 g had to be generated by the strips before use. All agents used were added directly to the chambers. All concentrations are reported as the final concentration in the chambers.

Several series of experiments were performed to examine the effects of chrysin on gallbladder strips. CCK (1 nM) was found to produce a stable long lasting tension after 3 min. This steady tension lasted at least 10 min [19,20]. In order to determine if chrysin could relax CCK- or KCl-induced tension, concentration response curves were generated. The CCK-induced tension was allowed to reach a steady level (3 min). The strips were exposed to 1 concentration of chrysin, the response was observed until the relaxation reached a steady level (approximately 5 min), the KHS was changed 3 times, and the strips were allowed to recover for 30 min. before testing a different concentration of chrysin. The concentration of chrysin (40 μM) was selected for use in subsequent experiments as it produced a reproducible relaxation. The same procedure was followed to generate a concentration response curve using 40 mM KCl instead of 1 nM CCK. The use of KCl to directly depolarize smooth muscle is a standard procedure.

In order to determine if the Ca^{2+} released from the endoplasmic reticulum mediated the chrysin-induced relaxation 2-APB (125 μM), a cell permeable inhibitor of IP_3-induced Ca^{2+} release, was added to the chambers 10 min prior to the CCK. The CCK was then added to the chambers. When the tension reached a steady level 40 μM chrysin was added to the chambers. The amount of relaxation was observed. The amount of relaxation observed when chrysin only was used was then compared to the amount of relaxation observed when the strips were treated with 2-APB and chrysin. This procedure was followed with each agent used.

When the PKA inhibitor PKA inhibitor 14–22 amide myristoylated (PKA-IM; 180 nM) was used, it was added to the chambers 15 min prior to CCK to ensure adequate time for entry into the smooth muscle. KT5823 (585 nM), a PKG inhibitor, was added to the chambers 5 min prior to the addition of CCK. Genistein (10 μM), a protein tyrosine kinase inhibitor, was added to the chambers 5 min prior to the addition of CCK.

The PKC inhibitors, chelerythrine Cl^- (5 μM) and bisindolymaleimide IV (BIM, 0.5 μM), were used together to determine the effects of PKC on chrysin-induced relaxation of CCK- or KCl-induced tension. They were added to the chambers 5 min prior to either CCK or KCl.

N^G-methyl-L-arginine acetate salt (L-NMMA; 20 μM) was used to determine if nitric oxide (NO) mediated the chrysin-induced relaxation. Fulvestrant (10 μM), an estrogen receptor blocker, was used to determine if chrysin might act via estrogen receptor activation. In addition, 17β-estradiol (E2, 50 μM) and chrysin were added to the chambers together to further determine if chrysin acted through the same receptors as E2 or was acting on some other receptor.

In order to determine if chrysin inhibited extracellular Ca^{2+} entry, 40 mM KCl was used to induce tension in the strips. After the amount of tension generated by the 40 mM KCl was recorded, the KHS was changed 3 times and the strips allowed to equilibrate for 25 min. The 40 μM chrysin was then added to the chambers 3 min prior to the addition of 40 mM KCl. The amount of tension generated was recorded and compared to that observed when the KCl was added to the chambers with no chrysin.

The following agents were purchased from Sigma (St. Louis, MO, USA): CCK, atropine, chrysin, L-NMMA, fulvestrant, E2, and bisindolymaleimide IV. The following agents were purchased from Calbiochem (LaJolla, CA, USA) PKA-IM, KT5823, chelerythrine Cl^-, genistein, and 2-APB. All agents were dissolved in either distilled water or dimethyl sulfoxide (DMSO). The amount of DMSO (10 μL) added to the chambers was determined to have no effect on the strips.

2.1. Statistical analysis

Statistical comparisons were done using either the paired *t*-test or analysis of variance with the Holm-Sidak test. Results are expressed as mean ± S.E. Differences among mean values with $p < 0.05$ were considered significant. The number of gallbladders (animals) used in each experiment are indicated by "n". Each gallbladder was used to prepare 4 strips.

3. Results

The analysis of the data follows methods reported previously [21,22]. Chrysin induced a concentration dependent relaxation in both CCK- and KCl-induced tension (Fig. 1). Chrysin, at all concentrations used, was observed to cause significantly ($p < 0.01$, $n = 16$) more relaxation in the CCK-induced tension than that induced by KCl. In order to determine if 1 nM CCK induced a similar amount of tension as 40 mM KCl, a comparison was made. KCl-induced significantly ($p < 0.01$) more tension (1.09 ± 0.11 g, $n = 15$) than CCK (0.88 ± 0.04 g, $n = 15$).

When the PKA/cAMP inhibitor PKA-IM was used, no significant effect was observed on the amount of CCK-induced tension. However, a significant ($p < 0.001$) decrease (69.8 ± 3.5% vs. 56.4 ± 2.8%, $n = 4$) in the amount of chrysin-induced relaxation was observed. (Fig. 2).

The use of 2-APB, an inhibitor of IP_3-induced Ca^{2+} release, caused a significant ($p < 0.001$) decrease in the amount of CCK-induced tension (0.87 ± 0.09 vs. 0.35 ± 0.06 g, $n = 4$; Fig. 3). When the