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Genistein reduces agonist-induced contractions of porcine coronary arterial smooth muscle in a cyclic AMP-dependent manner

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Abstract

Low concentrations of genistein enhance the vasodilatation induced by endothelium-independent vasodilators. The present study examined whether or not low concentrations of genistein modulate contractions in isolated porcine coronary arteries. The role of second messengers in the response to genistein was also assessed. Arterial rings were studied in organ baths and contracted with KCl, U-46619 (9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_2\alpha$), 5-hydroxytryptamine (5-HT) or endothelin-1 in the absence or presence of genistein (≤3 μM). Genistein significantly reduced agonist-induced but not KCl-induced contraction. Inhibition of endothelial nitric oxide synthase and disruption of endothelial function by Triton-X100 did not affect the modulation of contraction by genistein. The genistein-induced attenuation of contraction could be mimicked by both cAMP and cGMP analogs. However, only the cAMP-dependent protein kinase inhibitor, Rp-8-Br-cAMPS, abolished the effect of genistein. These results suggest that genistein reduces agonist-induced contraction by an endothelium-independent manner. This action is mediated via the cAMP-dependent signal transduction pathway.

Keywords: Genistein; Receptor-mediated contraction; Cyclic nucleotide; Coronary artery, porcine

1. Introduction

Genistein is a phytoestrogen structurally similar to 17β-estradiol and binds to both cytoplasmic and nuclear estrogen receptors at a rate of about 100–1000 times less than the hormone (Hsieh et al., 1998; Martin et al., 1978). Genistein binds to the estrogen receptor β to trigger many of the biological responses that are evoked by physiological levels of estrogens (Kuiper et al., 1998; Williams and Clarkson, 1998). In particular, significant reductions in serum total cholesterol, low-density lipoprotein (LDL) cholesterol and triglyceride without changes in serum high-density lipoprotein (HDL) cholesterol concentration were observed with soy protein, a rich source of genistein, in humans (Anderson et al., 1995; Hodgson et al., 1996; Mitchell and Collins, 1999). Expression of different types of estrogen receptors is tissue-specific and varies in affinities for different estrogen agonists. This heterogeneity helps to explain the variable responses observed in different tissues with any single-estrogen agonists (Matthews et al., 2000). A previous study demonstrated that acute treatment with 17β-estradiol reduced U46619-mediated contraction (Teoh and Man, 2000). A similar effect can be observed with other agonists [e.g., endothelin-1 and 5-hydroxytryptamine (5-HT)] but not potassium-chloride-induced contractions (Jiang et al., 1992). Little is known about the vascular effect exerted by other estrogenic compounds such as genistein (5,7,4-trihydroxyisoflavone).

Genistein is a well-established and effective nonselective tyrosine kinase inhibitor (Akiyama et al., 1987), and thus, may inhibit tyrosine kinase-mediated contraction of vascular smooth muscle (Liu and Sturek, 1996), in particular, the responses to 5-hydroxytryptamine (Watts et al., 1996). The cyclic nucleotides cAMP and cGMP shift the concentration–contraction curve to Ca^{2+} to the right in the rat mesenteric artery (Kawada et al., 1997). Cyclic nucleotides are also

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involved in the relaxing effect of genistein (Satake et al., 2000). However, it is unknown whether or not they mediate the inhibitory effect of genistein on vasoconstriction.

In the present study, the effects of genistein were investigated on the responses to various contractile agonists. The involvement of nitric oxide and other endothelial factors in the effect of genistein was determined. Finally, the contributions of tyrosine kinase inhibition and of the cAMP-/cGMP-dependent enzyme cascade were examined.

2. Material and methods

2.1. Drugs and chemicals

Genistein, bradykinin, endothelin-1, 5-hydroxytryptamine (5-HT), N^{ω}-nitro-L-arginine methyl ester (L-NAME) and tyrphostin 23 were products of Sigma (St. Louis, MO, USA). Sp-cAMPS, 8-Br-cAMP, 8-Br-cGMP, Rp-8-Br-cAMPS, Rp-8-Br-cGMPS and U46619 were purchased from Biomol (Plymouth Meeting, PA, USA). Triton-X100 was bought from Pharmacia Biotech (Uppssala, Sweden) and ethanol (absolute, EP) and dimethyl sulphoxide (DMSO) were purchased from Merck KgaA (Darmstadt, Germany). Stock solutions of U46619 (9,11-dideoxy-9α and 11α-methanoepoxy prostaglandin $F_2\alpha$) were dissolved in absolute ethanol, while genistein and tyrphostin 23 were prepared with DMSO. For the remaining drugs, distilled water was used. In the case of the final serial dilution, Krebs–Henseleit solution (KHS) was employed. Throughout the whole experiment, the DMSO and ethanol concentration in the bath would never exceed 0.03% (except in experiment involving tyrphostin 23 in which 0.3% DMSO was used) and 0.1%, respectively.

2.2. Tissue preparation

Pig hearts of either sex (50–80 kg body weight) were collected from a local abattoir and were transferred to the laboratory in cold oxygenated modified Krebs–Henseleit solution (KHS;, composition in mM: 120 NaCl, 4.76 KCl, 1.18 $MgSO_4$, 1.25 $CaCl_2$, 25 $NaHCO_3$, 1.18 NaH2PO_4 and 5.5 glucose). Pigs were processed according to the regulation laid down by Urban Services Department of the Government of Hong Kong Special Administrative Regions, China. Either the right or the left anterior descending coronary arteries were isolated and dissected free from adipose or connective tissues. The artery was then cut into 3-mm width rings. Rings were suspended immediately onto two stainless steel hooks. One hook was anchored into an organ bath filled with 5 ml of KHS. The other hook was connected to a force transducer (Model FT03, Grass Instrument Quincy, MA, USA) for the measurement of isometric tension. The bathing solution was continuously aerated with oxygen (95% O_2:5% CO_2) and maintained at 37 °C. The rings were allowed to equilibrate for 90 min before the experiments. The bathing solution was changed every 25 min and tension adjusted to 2 g except for the last 30 min. Vasomotor activities were displayed onto a screen by transforming the data points into voltage–time graph using a computer program (PICO Data Logger, Pico Technology Cambridge, UK).

After a 2-h equilibration period, the rings were contracted by changing the solution in the bath to a high potassium chloride solution (composition in mM: NaCl 95, KCl 30, $MgSO_4$ 1.18, $NaHCO_3$ 25, NaH_2PO_4 1.18, glucose 5.5 and $CaCl_2$ 1.25). The rings were then relaxed with bradykinin (1 μM). Only rings that contracted with more than 4 g and relaxed more than 40% of the original contraction were included in the study.

2.3. Effects of genistein on KCl-, U46619-, 5-hydroxytryptamine- and endothelin-1-induced contractions

After return to baseline level, rings were incubated with either genistein (3 μM, unless otherwise stated) or vehicle (0.03% DMSO) for 30 min before obtaining concentration–response curve to various contractile agonists. KCl (10–70 mM) was used to obtain concentration-dependent contraction via activation of voltage-gated Ca^{2+} channels. U46619 (0.1 nM–1 μM), 5-HT (10 nM–10 μM) and endothelin-1 (0.01–30 nM) were employed for receptor-mediated contraction.

2.4. Effects of L-NAME and Triton-X100 on contraction induced by U46619

To examine the involvement of nitric oxide synthase activity in the effect induced by genistein, 300 μM L-NAME (an inhibitor of nitric oxide synthase; Rees et al., 1990) was incubated together with genistein or the vehicle (DMSO, 0.03%) for 30 min before addition of increasing concentration of U46619. These concentration–contraction curves of U46619 were compared with those elicited in arterial rings without L-NAME treatments. The role of endothelial activity in contributing to genistein-induced effect was investigated in rings with normal and disrupted endothelium. To achieve disruption of the endothelium, 0.5% Triton-X100 was perfused through the porcine coronary artery at a rate of 1 ml per minute for 30 s before cutting into rings (Teoh et al., 2000).

2.5. Effects of tyrphostin 23 on U46619-induced contraction

Tyrphostin 23 is a nonspecific tyrosine kinase inhibitor, which interacts with the binding site of the enzyme (Watts et al., 1996). Direct relaxation of tyrphostin 23 (data not shown) on U46619-contracted coronary arterial rings suggested tyrphostin 23 at 30 μM carried similar direct relaxing effect as 3 μM genistein at similar condition.

Tyrphostin 23 (30 μM) was incubated for 30 min before U46619 concentration–response contraction was performed.

2.6. Involvement of cyclic nucleotides in the effect of genistein on U46619-induced contraction

Experiments were done to study the effects of cyclic nucleotides on U46619-mediated contraction in the presence of genistein. Three cyclic nucleotide analogs, 8-Br-cGMP (3 μM), 8-Br-cAMP (10 μM) and Sp-cAMPS (3 μM) were first introduced for 30 min. In separate rings, each analog was also incubated together with genistein for 30 min before obtaining U46619-induced contraction. Rp-8-Br-cAMPS (Gjertsen et al., 1995) and Rp-8-Br-cGMPS are highly potent protein kinase inhibitors that were selected to study the blocking effects on cAMP and cGMP pathway, respectively. Both Rp-8-Br-cAMPS (50 μM) and Rp-8-Br-cGMPS (10 μM) were given 20 min prior to 30-min treatment by genistein then followed by U46619-induced contraction.

2.7. Data and statistical analysis

Results were expressed as the mean±S.E.M., and *n* indicates the number of heart samples involved in the experiments. Contraction produced at a specific dose was expressed as the percentage (%) of the average of two KCl-mediated contractions. Analysis of variance (ANOVA) followed by either post hoc Bonferroni's test or Dunnett's test was applied to determine the differences between multiple groups of data using a computer statistical package (SPSS, SPSS Chicago, IL, USA). Differences between groups were regarded as significant when P was less than 0.05.

3. Results

3.1. Genistein

Genistein (3 μM) and the solvent (DMSO, 0.03%) had no significant effect on contractions to KCl (10–70 mM) (Fig. 1A). It shifted the concentration–response curves of

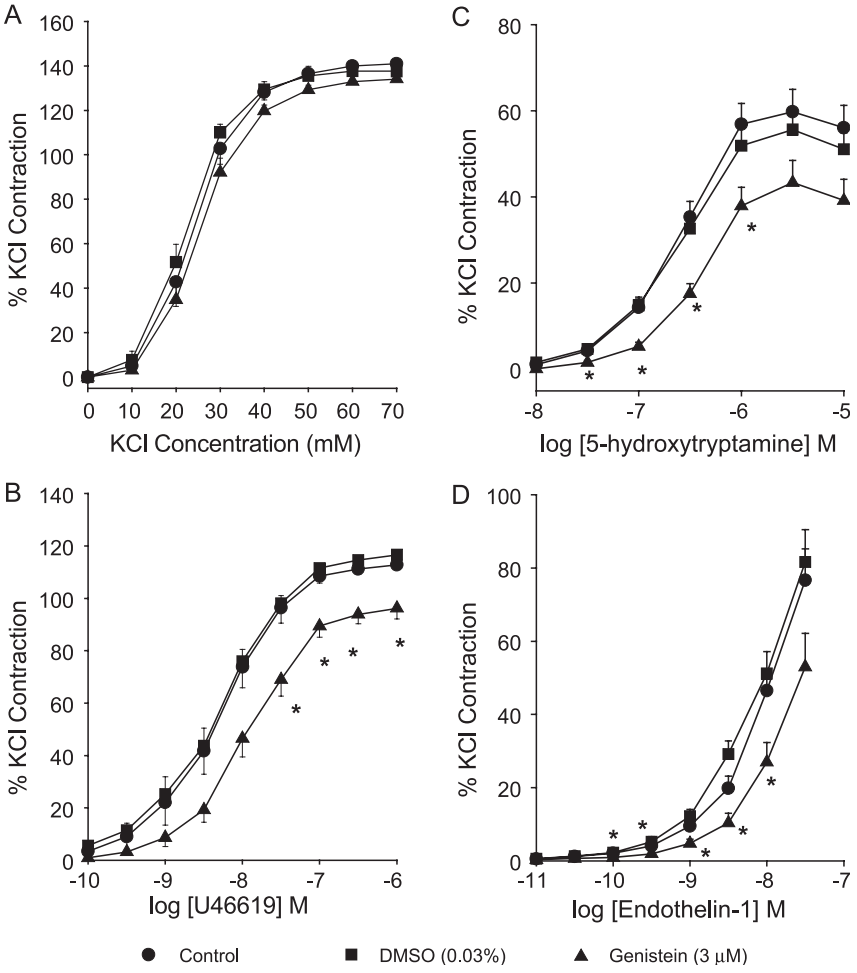

Fig. 1. Effects of genistein on (A) KCl-, (B) U46619-, (C) 5-hydroxytryptamine-, (D) endothelin-1-induced contractions in porcine coronary arterial rings treated either with genistein (▲), 0.03% DMSO (■) or none (●) for 30 min before the U46619-induced contraction. N=6–8. *P<0.05% vs. 0.03% DMSO.

U46619 (0.1 nM–1 μM), 5-HT (10 nM–10 μM) and endothelin-1 (0.01–30 nM) significantly to the right and reduced the maximal contraction to these agents (Fig. 1B, C and D; Table 1). DMSO (0.03%) had no significant effect.

3.2. Triton-X100 and L-NAME

Disruption of endothelium by Triton-X100 did not significantly alter the response to genistein (Fig. 2). Similar results were obtained using 5-HT (Fig. 3). L-NAME did not affect the ability of genistein to shift the concentration–response curve of U46619 to the right (Fig. 4). The EC_{50} to U46619 and 5-HT were raised by genistein in rings with or without Triton-X100 (Table 1). Genistein also significantly increased the EC_{50} to U46619 irrespective of the presence of L-NAME (Table 2).

3.3. Tyrphostin 23

The presence of tyrphostin 23 (30 μM) did not affect U46619-induced contraction when compared to the vehicle (DMSO, 0.3%) in porcine coronary artery (Fig. 5).

3.4. Cyclic nucleotides

Thirty-minute incubation of the three cyclic nucleotides, 8-Br-cGMP, 8-Br-cAMP and Sp-cAMPS at 3, 10 and 3 μM, respectively, significantly reduced the maximal contractions to U46619 (Fig. 6A, B and C). No additional effect was observed after simultaneous incubation with genistein plus the cyclic nucleotides. Rp-8-Br-cAMPS (50 μM) but not Rp-8-Br-cGMPS (10 μM) abolished the reduction of the U46619-induced contraction caused by genistein. Rp-8-Br-cAMPS or Rp-8-Br-cGMPS alone did not significantly affect the contractions (Fig. 7A and B).

Table 1
Effects of genistein on maximal contractions and EC_{50} to U46619, 5-hydroxytryptamine (5-HT) or endothelin-1 in porcine coronary arterial rings with or without endothelium

	Genistein concentration	Krebs–Henseleit perfused		Triton-X100 perfused (0.5%)	
		Maximal contraction (% KCl)	EC_{50} (nM)	Maximal contraction (% KCl)	EC_{50} (nM)
U46619	None	131±8	5.0	159±12	2.1
	3 μM	120±9	9.1[a]	153±11	4.4[a]
5-HT	None	56±5	280	87±4	138
	3 μM	45±8	621[a]	79±6	294[a]
Endothelin-1	None	82±9	ND	ND	ND
	3 μM	53±9[a]	ND	ND	ND

Endothelium was removed by the perfusion of 0.5% Triton-X100, and control studies were obtained using normal Krebs–Henseleit perfusion. Data represent mean±S.E.M. N=6–8 in each group. % KCl is the average of two KCl-induced contractions performed at the beginning of the experiment.
ND: not determined.
[a] P<0.05% vs. 0.03% DMSO.

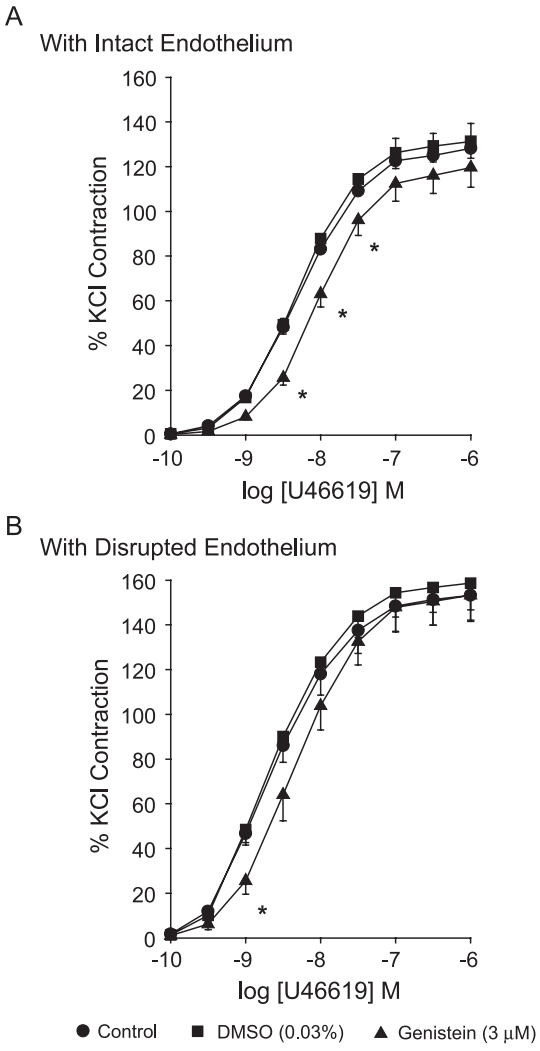

Fig. 2. Effects of genistein on U46619-induced contractions in rings (A) with or (B) without endothelium. The rings were treated either with genistein (▲), 3 μM), DMSO (■, 0.03%) or none (●) for 30 min before obtaining the concentration–response curve to U46619. N=5. *P<0.05% vs. 0.03% DMSO.

4. Discussion

The present study demonstrates the ability of genistein at nutritionally achievable levels (≤3 μM) to attenuate contraction in porcine coronary artery. At 3 μM, genistein is unlikely to exert a direct relaxing effect, and this concentration was hence selected to perform the present experiments (Lee and Man, 2003). Clinically, plasma concentration can be measured in the plasma of subjects consuming high soy diets (Setchell, 1998). U46619, 5-HT and endothelin-1 are contracting agents that elicit actions through receptor-mediated mechanism with the involvement of secondary messengers in the signal transduction pathway,

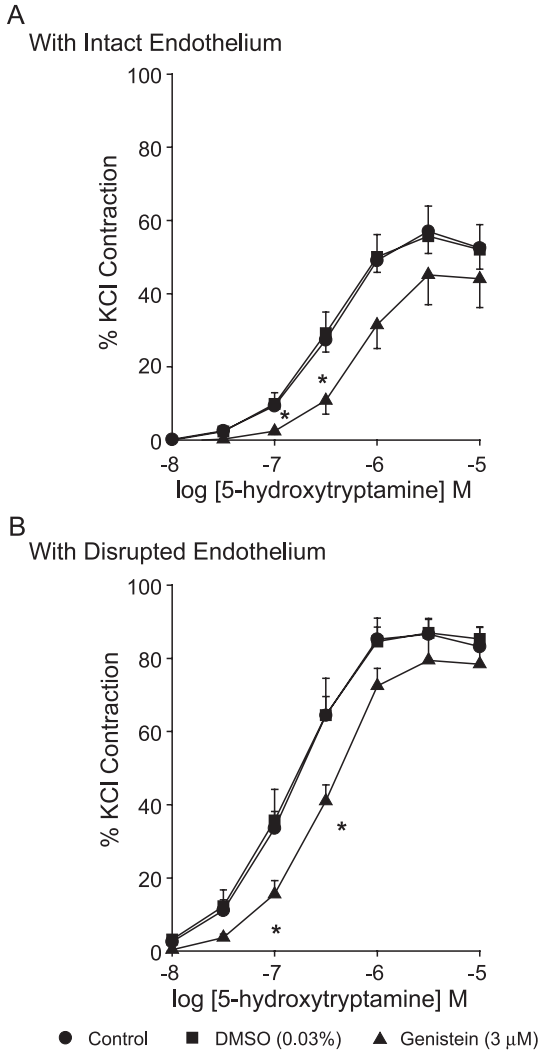

Fig. 3. Effects of genistein on 5-hydroxytryptamine-induced contractions in rings (A) with or (B) without endothelium. The rings were treated either with genistein (▲, 3 μM), DMSO (■, 0.03%) or none (●) for 30 min before obtaining the concentration–response curve to U46619. N=5. *P<0.05% vs. 0.03% DMSO.

while KCl contraction is voltage-gated through direct depolarization of the cell membrane (Yanagisawa and Okada, 1994). In the present study, the potency and efficacy of U46619, 5-HT and endothelin-1 in contracting porcine coronary arterial rings were reduced following short-term incubation (30 min) with genistein. By contrast, contraction to KCl was unaffected by the phytoestrogen. Similarly, high concentrations of genistein cause relaxation in the rat cerebral artery contracted with U46619 but not with KCl (Masumoto et al., 1997). Therefore, genistein does not affect directly the contractile process in vascular smooth muscle but exerts vascular effect by interfering with the activation of second messengers released by other stimuli.

The present results demonstrated that the inhibitory effect of genistein is preserved in the presence of L-NAME (300 μM). This suggests that the effect of genistein is independent of nitric oxide synthase activity. Moreover, genistein-induced inhibition can still be observed in the absence of the endothelium, despite an augmented contraction presumably due to the lack of basal release of endothelium-derived relaxing mediators. Thus, endothelium-derived vasoactive factors do not contribute to the vascular modulatory effect of genistein.

High concentrations of genistein may relax the U46619-contracted rat cerebral artery through inhibition of tyrosine kinase (Masumoto et al., 1997). Thus, the effect of the tyrosine kinase inhibitor, tyrphostin 23, was examined. The present data indicated that a low concentration of tyrphostin 23 (30 μM) with little effect on vascular contraction did not mimic the effect of genistein. Thus, it is unlikely that low concentration of genistein attenuates the responses of the porcine coronary artery by inhibiting tyrosine kinase activity.

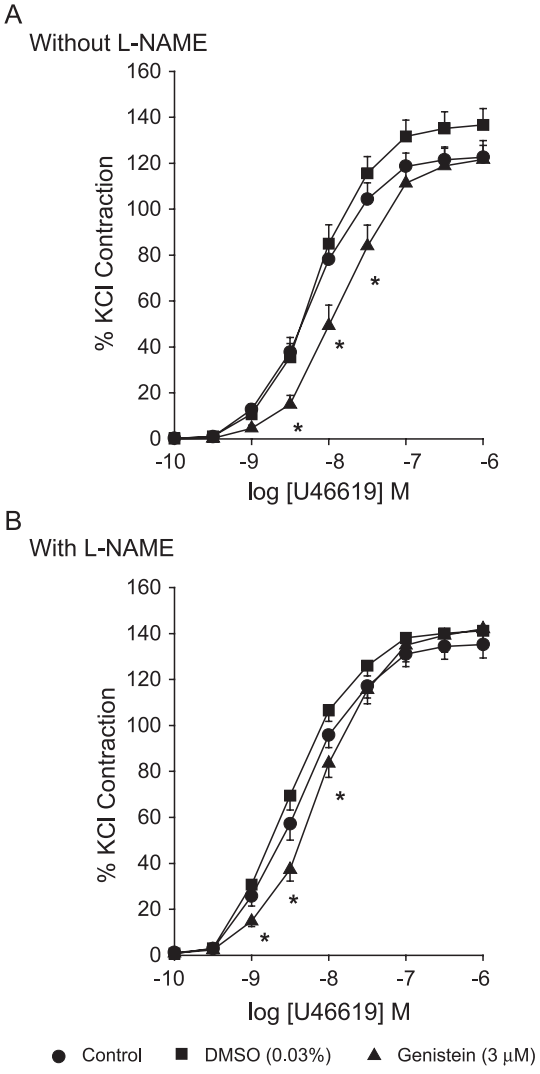

Fig. 4. Effects of genistein on U46619-induced contractions in the (A) absence or (B) presence of N^{ω}-nitro-L-arginine methyl ester (L-NAME). The rings treated either with genistein (▲, 3 μM), DMSO (■, 0.03%) or none (●) for 30 min before obtaining the concentration–response curve to contraction by U46619. N=8. *P<0.05% vs. 0.03% DMSO.

Table 2
Effects of genistein on maximal contractions and EC_{50} to U46619 in porcine coronary arterial rings in the absence and presence of L-NAME

	Genistein concentration	Without L-NAME		With L-NAME (300 μM)	
		Maximal contraction (% KCl)	EC_{50} (nM)	Maximal contraction (% KCl)	EC_{50} (nM)
U46619	None	137±7	6.9	141±5	2.7
	3 μM	122±8	14.9[a]	142±8	7.5[a]

Data represent mean±S.E.M. N=6–8 in each group. % KCl is the average of two KCl-induced contraction performed at the beginning of the experiment.
ND: not determined.
[a] P< 0.05% vs. 0.03% DMSO.

The production of cyclic nucleotides (both cAMP and cGMP) by receptor-mediated vasodilators is involved in the relaxation of vascular smooth muscle of rat mesenteric artery (Kawada et al., 1997). It is postulated that the formation of either cAMP or cGMP depends on types of agonists used and whether they activate cAMP- or cGMP-dependent protein kinases of a particular tissue type, respectively. Subsequently, the Ca^{2+} sensitivity and phosphorylation of contractile protein kinase, myosin light chain kinase, are reduced, and hence, the contraction diminishes (Kawada et al., 1997). In the present study, Sp-cAMPS, 8-Br-cAMP and 8-Br-cGMP reduce the maximal contraction to U46619. Meanwhile, simultaneous incubation with either cyclic nucleotide with genistein did not produce any additive inhibitory effect, suggesting that genistein may act through a similar pathway as the cyclic nucleotides to reduce contraction. This hypothesis was further confirmed with phosphorothioate stereoisomers of cAMP and cGMP (the Rp derivatives), which can inhibit the kinase activities by competing with the activating cyclic nucleotides for the cyclic nucleotide binding sites (De Wit et al., 1982). Indeed,

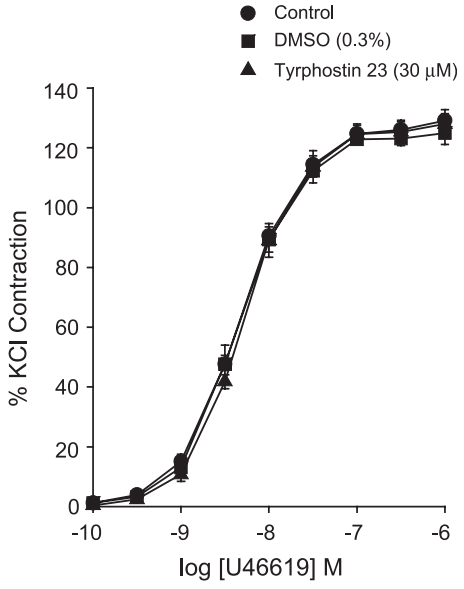

Fig. 5. Effects of tyrphostin 23 on U46619-induced contractions in porcine coronary arteries. The rings were treated either with tyrphostin 23 (▲, 30 μM), DMSO (■, 0.3%) or none (●) for 30 min before obtaining the concentration–response curve to U46619. N=4. *P<0.05% vs. 0.03% DMSO.

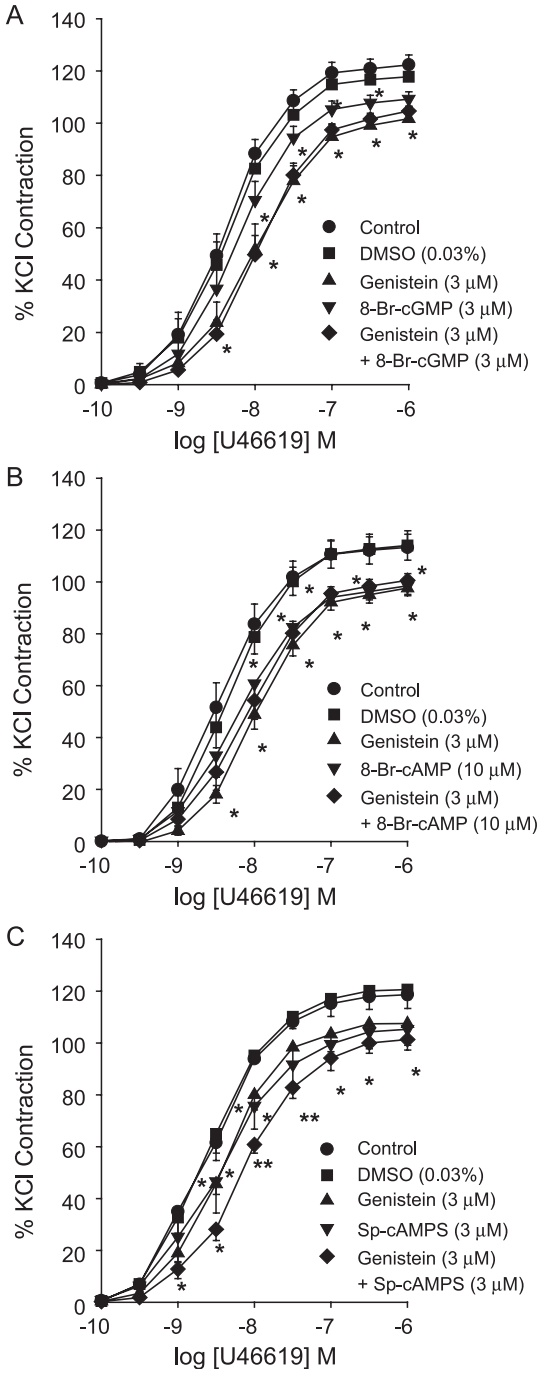

Fig. 6. Effects of (A) 8-Br-cGMP, (B) 8-Br-cAMP and (C) Sp-cAMPS plus genistein on U46619-induced contractions in porcine coronary arteries. The rings were treated either with (A) 8-Br-cGMP (▼, 3 μM), (B) 8-Br-cAMP (▼, 10 μM) and (C) Sp-cAMPS (▼, 3 μM), genistein (▲, 3 μM) or both (◆), DMSO (■, 0.03%) or none (●) for 30 min before obtaining the concentration–response curve to U46619. N=6–9. *P<0.05% vs. 0.03% DMSO.

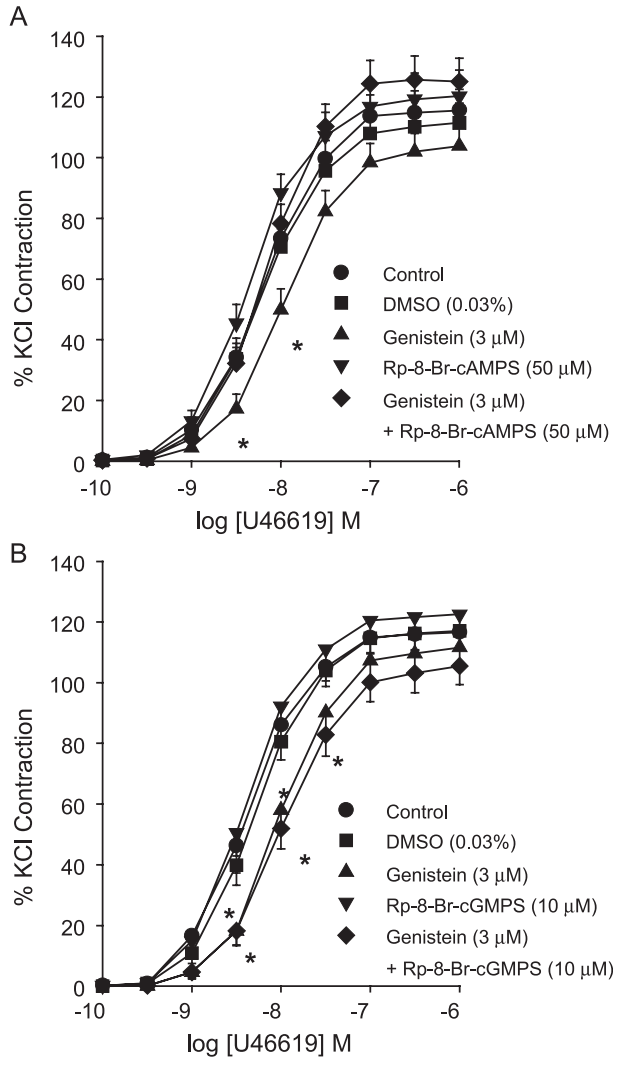

Fig. 7. Blocking effects of (A) Rp-8-Br-cAMPS and (B) 8-Br-cGMPS on genistein-mediated effect on U46619-induced contractions in porcine coronary arteries. The rings were treated with (A) Rp-8-Br-cAMPS (▼, 50 μM) or (B) Rp-8-Br-cGMPS (▼, 10 μM) for 20 min. Then, they were exposed to either genistein (▲, 3 μM), DMSO (■, 0.03%), none (●) or genistein with (A) Rp-8-Br-cAMPS or (B) Rp-8-Br-cGMPS (◆) for 30 min before obtaining the concentration–response curve to U46619. N=7–8. *P<0.05% vs. 0.03% DMSO.

blockade of the genistein-induced effect was observed with Rp-8-Br-cAMPS. This reveals the involvement of the cAMP-dependent pathway in the reduction of contraction caused by genistein.

Unlike Rp-8-Br-cAMPS, Rp-8-Br-cGMPS did not affect the effect of genistein on U46619-induced contractions. This result appears contradictory to the observation that the cGMP analogue, 8-Br-cGMP, mimics the action of genistein. The role of the cGMP-dependent enzyme cascade in producing the genistein-induced reduction of contraction is therefore unclear. One possible explanation is that cGMP increases the cAMP levels by inhibiting the activity of the phosphodiesterase that breaks down cAMP (Polson and Strada, 1996; Satake et al., 1995). On the other hand, total cAMP phosphodiesterase activity in rat cultured aortic vascular smooth muscle cells is not affected by 8-Br-cGMP (Rose et al., 1997). Alternatively, the phenomenon of cross-talk between cyclic nucleotides and their respective protein kinases may account for the present observation of the apparent involvement of cGMP but not of its protein kinase in the modulatory action of genistein (Dhanakoti et al., 2000; Kawada et al., 1997; Millard et al., 1998; Ruiz-Velasco et al., 1998; Taguchi et al., 1997; Tewari and Simard, 1997; White et al., 2000). There is evidence of crossover activation of cAMP-dependent protein kinase by cGMP in vascular smooth muscle (Cornwell et al., 1994; Ruiz-Velasco et al., 1998). Indeed, cAMP-dependent protein kinase is activated by nitric oxide, which causes increase in cGMP level (Tsukada et al., 2002). As such, the present results indicate a novel mechanism for genistein to exert its modulatory effect through the second messenger cAMP and/or cGMP with the resultant activation of cAMP-dependent protein kinase. Further studies are needed to elucidate these pathways in more details.

In conclusion, genistein, at 3 μM, inhibits receptor-mediated contractions but not depolarization-induced contraction. The inhibitory effect of genistein does not involve nitric oxide synthase or other endothelium-derived factors. This effect is also not mimicked by a tyrosine kinase inhibitor. The attenuation of agonist-induced contraction by genistein acts through the cAMP-dependent signal transduction pathway.

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