Pulmonary, gastrointestinal and urogenital pharmacology

Relaxant mechanisms of 3, 5, 7, 3', 4'-pentamethoxyflavone on isolated human cavernosum

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A B S T R A C T
We have investigated effects and mechanisms responsible for the activity of 3, 5, 7, 3’, 4’-pentamethoxyflavone (PMF) on isolated human cavernosum. PMF is the major flavone isolated from Kaempferia parviflora claimed to act as an aphrodisiac. PMF caused relaxation of phenylephrine precontracted human cavernosal strips, and this effect was slightly inhibited by Nω-nitro-ω-arginine, a nitric oxide synthase inhibitor, but not by ODQ (soluble guanylate cyclase inhibitor), TEA (tetraethylammonium, blocker of voltage-dependent K+ channels) or glybenclamide (blocker of ATP-dependent K+ channels). PMF did not significantly inhibit the relaxant activity of glyceryl trinitrate on human cavernosal strips precontracted with phenylephrine. In contrast, sildenafil (phosphodiesterase inhibitor) potentiated the relaxant activity of glyceryl trinitrate but not of acetylcholine. In normal Krebs solution with nifedipine (blocker of L-type Ca2+ channels), or in Ca2+-free Krebs solution, PMF caused a further inhibition of human cavernosum contracted with phenylephrine. In human cavernosum treated with thapsigargin (inhibitor of sarcoplasmic reticulum Ca2+-ATPase) in Ca2+-free Krebs solution, PMF suppressed the concentration-response curve of human cavernosum to phenylephrine and a further suppression was found when SKF-96365 (a blocker of store-operated Ca2+ channels) and Y-27632 (inhibitor of Rho-kinase), but not nifedipine, were added sequentially. Thus, PMF had only a weak effect on the release of nitric oxide, and had no effect as a KATP- or KCa channel opener, a phosphodiesterase inhibitor, a store-operated Ca2+ channel blocker or a Rho-kinase inhibitor. Therefore, these studies suggest that PMF causes relaxation of human cavernosum through voltage-dependent Ca2+ channels and other mechanisms associated with calcium mobilization.

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1. Introduction
Erectile dysfunction has multifactorial causes (Andersson, 2001; Solomon et al., 2003; Virag et al., 1985), but all lead to an impaired relaxation of the corpus cavernosum or the penile arteries (Andersson, 2011). A number of drugs have been developed for treatment of erectile dysfunction. So far the phosphodiesterase type 5 (PDE5) inhibitor, sildenafil, and its derivatives are the most successful for management of erectile dysfunction, and are commonly used for first line therapy (Hatzimouratidis and Hatzichristou, 2008). However, PDE5 inhibitors require sexual stimulation to induce nitric oxide release, enzymatic production of cGMP and finally erection (Boolell et al., 1996; Lin et al., 2003; Russell et al., 2004). Furthermore, some forms of erectile dysfunction involve changes in the control of nitric oxide synthesis (e.g. atherosclerosis, diabetes, ageing: Maas et al., 2002, 2005; Ioakeimidis et al., 2011; Park et al., 2009; Aktoz et al., 2010; Toda, 2012). In such situations PDE5 inhibition would have limited therapeutic effect treatment (Malavige and Levy, 2009). Therefore a drug having direct relaxation effect on corpus cavernosum may be another effective approach for the treatment of erectile dysfunction.

Flavonoids, polyphenolic compounds found in dietary sources have pharmacological activities. In particular, quercetin – the most abundant flavonoid in the human diet – is widely distributed throughout the vegetables and fruits used by humans (Boots et al., 2008). Quercetin is claimed to exert beneficial health effects on cardiovascular disease (Perez-Vizcaino et al., 2009). Consistently, chronic administration of quercetin reduces blood pressure in spontaneously hypertensive rats (Duarte et al., 2001a, 2001b) and other rat models of hypertension (Perez-Vizcaino et al., 2009). Erectile dysfunction is an early symptom of cardiovascular disease, and both share similar pathogenic involvement of dysfunctional endothelium (Jackson et al., 2006; Montorsi et al., 2006; Russell et al., 2004; Wespes and Schulman, 2010). In agreement, Zhang et al. (2011) found that 8-week administration of quercetin (50 mg/kg) ameliorated erectile function in streptozotocin-induced diabetic rats.

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Several studies have investigated the mechanisms of quercetin. In an in vitro study, quercetin has endothelium-independent vasodilatory effects (Duarte et al., 1993a, 1993b). In addition, Hnatyszyn et al. (2004) found that quercetin and its methoxylated form: quercetin 3, 7, 3', 4'-tetramethylether and quercetin 3, 5, 7, 3', 4'-pentamethylether, caused similar relaxation of isolated guinea-pig cavernosum. However, the mechanism responsible for this effect is not yet established. Interestingly, quercetin 3, 5, 7, 3', 4'-pentamethylether (3, 5, 7, 3', 4'-pentamethoxyflavone) is found in rhizomes of Kaempferia parviflora, a plant used in Thai traditional medicine especially by the Mong people in northern Thailand, for sexual enhancement and treatment of erectile dysfunction (Yenjai et al., 2004). We hypothesized that 3, 5, 7, 3', 4'-pentamethoxyflavone (PMF) isolated from rhizomes of K. parviflora would cause relaxation of isolated human cavernosum obtained from patients undergoing a sex change operation. We also investigated possible mechanisms of any relaxation effect. The results would in part test the claims that extracts from K. parviflora could treat erectile dysfunction and be used to stimulate sexual activity in men.

2. Materials and methods

2.1. Plant material

Fresh rhizomes of K. parviflora were collected in Ampor Phurua, Loei Province, Thailand. Authentication was achieved by comparison with the herbarium specimen in the Department of Biology Herbarium, Faculty of Science, Prince of Songkla University, Thailand, where a voucher specimen (Collecting no. 2548-03) of the plant material has been deposited.

2.2. Extraction and isolation of the 3, 5, 7, 3', 4'-pentamethoxyflavone (PMF) from rhizomes of Kaempferia parviflora

The fresh rhizomes of K. parviflora (20 kg) were blended and extracted successively by maceration three times with 95% ethanol (3 × 20 l) for a week. The ethanol soluble part was filtered and evaporated under reduced pressure. The ethanol extract residue was lyophilized to obtain a black powder of the crude K. parviflora ethanol extract (KPE) with a yield of 520 g.

Using human cavernosum relaxant guided fractionation, the KPE extract (500 g) was subjected to column chromatography over silica gel 100 (0.063–0.200 mm, 850 g) and eluted with a gradient of CHCl3:MeOH from 100% CHCl3 to 100% MeOH, to yield 6 fractions (A1–A6). On the basis of thin layer chromatography (TLC: gel 60F254 Al sheets, Merck, detection at 254 and 365 nm, and CHCl3:MeOH, 8:2 (v:v) as the mobile phase the fractions with relaxant activity, A3 and A4, were re-chromatographed on silica gel 60 (0.040–0.063 mm) and eluted with a gradient of CHCl3:MeOH from 100% CHCl3 to 100% MeOH, yielding 6 more fractions (B1–B6). The fraction with relaxant activity, B4 was further fractionated by silica gel reversed phase C18 column chromatography using a gradient elution of MeOH–H2O: from 30% MeOH to 100% MeOH increasing each step by 10% MeOH and using 3.5 l of each concentration. This yielded a pure compound that was identified as 3, 5, 7, 3', 4'-pentamethoxyflavone (PMF, 2.8 g, 99% pure). The purity of the isolated active compound was analyzed by high performance liquid chromatography (HPLC). Analytical HPLC was carried out on a HP 1100 system equipped with a photodiode array detector (Agilent Technologies). The compound was analyzed on a SymmetryC18 column (5 μm, 3.9 × 150 mm i.d.; Waters), with a gradient of CH3OH:H2O+0.05% of trifluoroacetic acid (5:95→100:0). The flow rate was 1 ml/min; the UV traces were measured at 210 and 254 nm and UV spectra (DAD) were recorded between 190 and 500 nm.

The structures of the pure compounds (Fig. 1) were characterized by spectroscopic data. 1H and 13C NMR spectra were recorded on a 300 MHz Bruker FTNMR Ultra Shield spectrometer in deuterochloroform solution with tetramethylsilane as an internal standard and comparison with the literature (Dong et al., 1999) and standard. Their chemical structures and HPLC chromatograms together with the UV spectrum of the PMF are shown in Fig. 1.

![Chemical structure and HPLC chromatogram together with the UV spectrum of 3, 5, 7, 3', 4'-pentamethoxyflavone (PMF).](image-url)
2.3. Pharmacological studies

2.3.1. Preparation of human cavernosal strip

Cavernosal tissue was obtained from potent men who had undergone a sex change operation aged 22–35 years, from a total of 40 patients. All the patients were informed and gave their written consent. The experimental protocol was approved by the Prince of Songkla University Ethical Committee.

Preparation of human cavernous strips was modified from Mirone et al. (2000). The amputated penis was carefully placed in cold oxygenated Krebs Henseleit solution and washed immediately twice with the same solution. The penis was carefully opened along the median section, and longitudinal strips of about 0.2 × 0.2 × 0.5 mm (width × thick × long) were dissected following the penis trabecular structure. The human cavernosal strip was mounted in a 20-ml organ bath, one end was fixed at the bottom and the other end connected to a force-displacement transducer (FT03C) connected to a Grass polygraph, under a basal tension of 1.0 g. The human cavernosal strip was allowed to equilibrate for 60 min with changes in Krebs Henseleit solution every 15 min.

1.0 g. The human cavernosal strip was allowed to equilibrate for 10 min, and the relaxant response to 10 μM acetylcholine was recorded. The experiment was continued with that strip only when the relaxation response obtained was more than 80%. Each set of human cavernosal strips was used for only one drug.

2.3.2. Experimental protocol

2.3.2.1. Inhibition of nitric oxide stimulator, guanylate cyclase and K⁺ channels. After equilibration, the human cavernosal strip was precontracted with 1 μM phenylephrine for 10 min (plateau reached), and the cumulative concentration–response (C–R) relationships of the human cavernosal strip to PMF (0.01–3 mM) were determined. Following several washings and re-equilibration of the human cavernosal strip for another 60 min with changes in Krebs Henseleit solution every 15 min, the human cavernosal strips were incubated with N²-nitro–arginine (LNA, 0.3 mM, inhibitor of nitric oxide synthase), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10 μM, a soluble guanylate cyclase inhibitor), tetraethylammonium (TEA, 1 mM, a voltage activated K⁺ channel blocker) or glibenclamide (10 μM, an ATP sensitive K⁺ channel inhibitor) for 40 min. Then the PMF cumulative C–R relationship was determined on the phenylephrine-induced contraction in the continuous presence of each drug.

2.3.2.2. Inhibition of phosphodiesterase activity. Another set of human cavernosal strips was used to study the phosphodiesterase 5 inhibitory activity. After equilibration, a cumulative C–R relationship to acetylcholine or to glycerol trinitrate was determined on the phenylephrine-induced contraction (1 μM). Following several washings, and re-equilibration for 60 min, the human cavernosal strip was incubated with sildenafil (1 and 10 μM, phosphodiesterase type 5 and 6 inhibitor) or PMF (0.1 and 0.3 mM) for 40 min. Then the C–R relationship to the acetylcholine or to the glycerol trinitrate on the human cavernosal strip was determined on the phenylephrine-induced contraction (1 μM) in the continuous presence of each concentration of each drug.

2.3.2.3. Inhibition of voltage-dependent calcium channels, intracellular Ca²⁺ mobilization, store-operated calcium channels, and Rho-kinase. In all the experiments described in this section, human cavernosal strips were first equilibrated for 60 min in Krebs solution, followed by functional assessment of the endothelium as above, after which they were incubated with LNA for 60 min with a replacement of the incubation medium every 20 min. Thereafter LNA was present throughout.

To determine whether PMF plays a role as a voltage-dependent Ca²⁺ channel blocker, the contractile response (10 min) to phenylephrine (1 mM) was studied in the normal Krebs medium. This was followed by several washings and re-equilibration for 40 min, then the human cavernosal strips were incubated with nifedipine (3 μM) for 20 min and again the contractile response to 1 μM phenylephrine was determined after which the cumulative phenylephrine C–R relationship was studied in the presence of nifedipine. The same procedure was repeated in the presence of nifedipine with PMF (0.3 mM).

To determine whether PMF inhibits intracellular Ca²⁺ mobilization, a similar protocol was carried out with another set of the human cavernosal strips in the Ca²⁺-free Krebs solution before and after incubating the human cavernosal strips with PMF.

To confirm whether PMF plays a role as a Ca²⁺ channel blocker, or is involved in the myofilament Ca²⁺ sensitization via the Rho-kinase pathway, the human cavernosal strips were challenged with 1 μM phenylephrine or 80 mM KCl for 20 min. Following several washing, and re-equilibration for 40 min, the human cavernosal strips were then incubated with nifedipine (0.01–100 μM), Y-27632 (0.3–30 μM, a Rho-kinase inhibitor) or PMF (0.01–0.3 mM) for 20 min, then challenged with 1 μM phenylephrine or 80 mM KCl for 20 min. This procedure was repeated at each concentration of each incubating substance.

To determine the role of intracellular calcium stores, for other sets of human cavernosal strips, the human cavernosal strips were challenged with 1 μM phenylephrine followed by several washings and re-equilibration for another 40 min. The human cavernosal strips were then incubated with thapsigargin (3 μM), a sarcoplasmic reticulum ATPase inhibitor, for 40 min by which time the contraction of the human cavernosal strips had reached a plateau. Then PMF (0.3 mM), SKF-96365 (100 μM) or Y-27632 (30 μM) were added and incubated for 20 min, at which time the human cavernosal strip had relaxed to its maximal level. Then a cumulative C–R relationship to phenylephrine was obtained in the presence of thapsigargin together with PMF, SKF-96365 or Y-27632. A similar protocol was used in a separate set of human cavernosal strips after incubating the human cavernosal strips with thapsigargin for 40 min, the human cavernosal strip was incubated with nifedipine for 5 min, then Y-27632, SKF-96365 or PMF was added and incubated for 20 min and the C–R relationships to phenylephrine were obtained in the presence of their corresponding cocktails.

To determine the effects of extracellular calcium, for the final 20 min of the re-equilibration process of the human cavernosal strips in LNA, strips were exposed to Ca²⁺-free Krebs solution (in LNA) for 20 min. The cumulative C–R relationship to phenylephrine was determined, followed by several washings and a re-equilibration for 40 min. Then the medium was replaced with Ca²⁺-free Krebs solution with 0.5 mM EGTA for 20 min then incubated with thapsigargin (3 μM) for 40 min to deplete intracellular Ca²⁺ stores in the sarcoplasmic reticulum. Then the human cavernosal strips were replaced with Ca²⁺-free Krebs solution without EGTA and the C–R relationships to phenylephrine were performed in the absence or presence of PMF alone or in combination with nifedipine, SKF-96365 and/or Y-27632 as required.

2.4. Drugs

The organ bath contained the Krebs–Henseleit solution of the following composition (mM) NaCl 118.3, KCl 4.7, CaCl₂ 1.9, MgSO₄·7H₂O 0.45, KH₂PO₄ 1.18, NaHCO₃ 25.0, glucose 11.66, Na₂EDTA 0.024 and ascorbic acid 0.09, maintained at 37 °C, was
continuously bubbled with 95% O2 and CO2. In the Ca\(^{2+}\)-free Krebs solution, the 1.9 mM CaCl\(_2\) was omitted and EGTA 0.5 mM was added as required.

The following drugs were used: acetylcholine chloride, nifedipine, N\(^6\)-nitro-L-arginine (LNA), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ), phenylephrine hydrochloride and tetrathyrammonium (TEA) were from Sigma, U.S.A. Thapsigargin, SKF-96365 and trans-4-[[1-(1H)-1-aminoethyl]-N-4-pyridinylcyclohexanecarboxamide dihydrochloride (Y-27632) were from Trocis, UK and sildenafil citrate (Viagra) was from Pfizer. LNA, thapsigargin, SKF-96365, L-27632 and sildenafil were dissolved in distilled water, nifedipine was dissolved in 20% DMSO, and the remainder was dissolved in a solution (1 l) containing NaCl 9 g, NaH\(_2\)PO\(_4\) 0.19 g and ascorbic acid 0.03 g.

2.5. Statistical analysis

Results are expressed as a mean ± S.E.M. where n indicates the number of human cavernosal strips. Each strip was obtained from a different patient. Drug-induced relaxation was measured as the decline from the maximal steady tension produced by phenylephrine (1 mM). The steady decline achieved at each drug concentration was expressed as a percentage of the initial maximum produced by phenylephrine (1 mM). Contraction induced by phenylephrine (1 mM) both in normal Krebs and Ca\(^{2+}\)-free Krebs solution was expressed as grams of the phasic contraction (1–2 min). Contractions induced by thapsigargin (3 mM) in normal Krebs solution with and without any drugs were expressed as a percentage of the maximum phasic contraction (1–2 min) induced by 1 mM phenylephrine. Contractions induced by phenylephrine (1 mM) or KCl (80 mM) alone or in the presence of any drugs for different times (1–20 min) were also expressed as a percentage of the maximal phasic contraction of their corresponding control. The slopes of the phasic contraction produced by phenylephrine (1 mM) and KCl (80 mM) were calculated from the linear portion of the curve (0–1 min) using a regression line analysis computer program (Microsoft Office Excel 2007). The contractile cumulative C–R curve to phenylephrine, and the steady increase achieved at each phenylephrine concentration was expressed as a percentage of the Emax obtained from their control group. Statistical comparisons were performed using the Student’s two-tailed t-test for paired or unpaired data. In all cases, a P value < 0.05 was considered to be statistically significant.

3. Results

3.1. Nitric oxide, guanylate cyclase, K\(^+\) channels

PMF caused relaxation of human cavernosal strips precontracted with phenylephrine in a concentration-dependent manner. Only low concentrations (3 and 10 μM) of the PMF were significantly inhibited by LNA (0.3 mM). At higher concentrations of PMF, the relaxation of the human cavernosal strip was similar to that of the control group (Fig. 2). Neither ODQ (10 μM), TEA (1 mM) nor glybenclamide (10 μM) had significant effect on the relaxant activity of the PMF (data not shown).

3.2. Inhibition of phosphodiesterase activity

Acetylcholine and glyceryl trinitrate caused relaxation of the human cavernosal strip precontracted with phenylephrine (1 μM) in a concentration-dependent manner. Incubating for 20 min with PMF (0.1 and 0.3 mM) or sildenafil (1 and 10 μM) did not significantly modify the relaxation of the C–R curve of the human cavernosal strip to acetylcholine (Fig. 3A and B). Sildenafil, but not PMF potentiated the relaxant activity of the human cavernosal strip to the glyceryl trinitrate (Fig. 3C and D).

3.3. Further experiments

In order to remove any effects of nitric oxide from nitric oxide synthase, further experiments were studied after having incubated the human cavernosal strip with LNA, a nitric oxide synthase inhibitor, for 40 min, the experiments being performed in the continued presence of LNA.

3.3.1. Blocking of voltage-dependent calcium channels

Nifedipine (3 μM) or a Ca\(^{2+}\)-free Krebs solution significantly inhibited the contractile responses of the human cavernosal strips to phenylephrine (1 μM). When PMF was also added a further inhibition was obtained (Table 1).

Effects of nifedipine, Y-27632 or PMF on the contractile response to phenylephrine and KCl are shown in Fig. 4, and the slopes of the phasic contractions are shown in Tables 2a–2c. In the presence of nifedipine, both the slopes of the phasic contractions and the amplitude of the tonic contraction produced by 1 μM phenylephrine were depressed. Maximal inhibition of about 70% was obtained with 1 μM nifedipine. In the case of the contraction to KCl, the maximal inhibition by nifedipine was about 75% at a concentration of 1 μM (Fig. 4A and B). Y-27632 caused an inhibition of both the slopes of the phasic contractions and the amplitude of the tonic contraction of the human cavernosal strips to both phenylephrine and KCl in a concentration-dependent manner (Fig. 4C and D). PMF caused concentration-dependent decreases in both the slopes of the phasic contractions and the amplitude of the tonic contraction of the human cavernosal strips to both phenylephrine and KCl (Fig. 4E and F).

A further experiment was designed to investigate whether PMF plays a role as an inhibitor of intracellular Ca\(^{2+}\) mobilization. In the normal Krebs solution in the presence of LNA and nifedipine, Y-27632 and PMF, but not SKF-96365, decreased the contractile responses of the human cavernosal strips to phenylephrine. In the Ca\(^{2+}\)-free Krebs solution, on the other hand, Y-27632 completely inhibited the C–R curve of the human cavernosal strips to the phenylephrine. SKF-96365 caused a slight...
conditions where store-operated Ca\(^{2+}\) channels are activated.

Thapsigargin (3 \(\mu\)M), a SERCA pump inhibitor, to reduce the Ca\(^{2+}\) in the sarcoplasmic reticulum and this in turn stimulated the opening of the plasma membrane store-operated Ca\(^{2+}\) channels (Noguera et al., 1998; Quinn et al., 2004). In the normal Krebs solution in the presence of LNA, incubating the human cavernosal strips with thapsigargin (3 \(\mu\)M) resulted in a contraction of the human cavernosal strip that developed slowly and reached its maximum at about the same level as that produced by phenylephrine (1 \(\mu\)M) and reached a plateau by 40 min (Table 3). At the plateau stage when SKF-96365 (100 \(\mu\)M) was also added into the incubation medium, complete relaxation of the human cavernosal strips was found. Y-27632 (30 \(\mu\)M) or PMF (0.3 \(\mu\)M) also caused near maximal relaxation (Table 3, left). Nifedipine caused relaxation of the human cavernosal strip precontracted with thapsigargin reaching a maximal relaxation of about 32%. In the presence of nifedipine, Y-27632, SKF-96365 or PMF there was a further relaxation of the human cavernosal strips precontracted with thapsigargin to the same levels as they were without nifedipine (Table 3, right).

In the Ca\(^{2+}\)-free Krebs solution with LNA and EGTA, incubating the human cavernosal strip for 40 min with thapsigargin (3 \(\mu\)M) produced no contraction of the human cavernosal strip (data not shown). After washing the human cavernosal strip with Ca\(^{2+}\)-free (LNA) Krebs solution without EGTA, following just thapsigargin pre-treatment, phenylephrine caused a contraction of the human cavernosal strip in a concentration-dependent manner. However, the maximal contraction to phenylephrine obtained from the thapsigargin pretreated human cavernosal strip was significantly lower than the one without thapsigargin pretreatment (Fig. 6).

The phenylephrine induced C–R curve of the thapsigargin pretreated human cavernosal strip in the Ca\(^{2+}\)-free Krebs solution, presumably involves Ca\(^{2+}\) release from thapsigargin-insensitive (IP\(_3\)) Ca\(^{2+}\)-stores and possibly Rho-kinase activation. In the Ca\(^{2+}\)-free medium without EGTA, nifedipine caused a significant shift of the C–R curve to phenylephrine to the right (Fig. 7A). It was found that Y-27632 alone (Fig. 7B) or in the presence of nifedipine (Fig. 7C) and/or SKF-96365 (Fig. 7D–G) almost completely inhibited the C–R curve of the human cavernosal strips to phenylephrine. SKF-96365 alone caused a significant inhibition of the C–R curve of the human cavernosal strip to phenylephrine and this effect was not further inhibited after adding nifedipine (Fig. 7D and E). PMF significantly inhibited the C–R curve of the

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**Table 1**

Effects of nifedipine (Nife, 3 \(\mu\)M), Ca\(^{2+}\)-free Krebs solution (Ca\(^{2+}\)-free) and/or 3, 5, 7, 3’, 4-pentamethoxyflavone (PMF, 0.3 mM) on the phasic contractile responses of the human cavernosum to phenylephrine (Phe, 1 \(\mu\)M).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Contraction (g)</th>
<th>Treatment</th>
<th>Contraction (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe (control)</td>
<td>7.3 ± 1.03</td>
<td>Phe (control)</td>
<td>5.53 ± 0.89</td>
</tr>
<tr>
<td>Nife + Phe</td>
<td>1.75 ± 0.52</td>
<td>Ca(^{2+})-free + Phe</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Nife + PMF + Phe</td>
<td>0.10 ± 0.01b</td>
<td>Ca(^{2+})-free + PMF + Phe</td>
<td>0.02 ± 0.01b</td>
</tr>
</tbody>
</table>

Data represents a mean ± S.E.M. of 6 human cavernosal strips each obtained from a different person.

- a Significantly lower than the control group, \(P < 0.05\).
- b Significantly lower than their corresponding control group and the one with nifedipine or in Ca\(^{2+}\)-free medium, \(P < 0.05\).

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**Fig. 3.** Effects of sildenafil (Silden) or 3, 5, 7, 3’, 4-pentamethoxyflavone (PMF) on relaxation of the phenylephrine (1 \(\mu\)M) precontracted human cavernosum to acetylcholine or glyceryl trinitrate. Drug-relaxation is expressed as a percentage inhibition of the maximum phenylephrine tension. Each point represents a mean ± S.E.M. of 6 human cavernosal strips each obtained from a different person. (\(^*\)Significantly lower than the control (\(\sim\)) group, \(P < 0.05\).
phenylephrine, but when nifedipine was also added no further inhibition was obtained (Fig. 7F). However, when SKF-96365 and Y-27632 were also added sequentially, a further inhibition was observed at each step (Fig. 7F). In human cavernosal strips incubated with nifedipine and SKF-96365 and Y-27632, PMF has unexpectedly a slight contractile effect (Fig. 7G).

Table 2a
Effect of nifedipine (20 min incubation for each concentration) on the contractile response of the human cavernosal strips to phenylephrine (Phe, 1 μM) or KCl (80 mM).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Responses to Phe</th>
<th>Responses to KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>92.91 ± 2.18</td>
<td>88.43 ± 5.84</td>
</tr>
<tr>
<td>0.01 μM Nifedipine</td>
<td>72.46 ± 1.32</td>
<td>48.64 ± 10.64</td>
</tr>
<tr>
<td>0.1 μM Nifedipine</td>
<td>54.02 ± 6.65</td>
<td>23.53 ± 6.28</td>
</tr>
<tr>
<td>1 μM Nifedipine</td>
<td>29.04 ± 4.43</td>
<td>17.25 ± 1.92</td>
</tr>
<tr>
<td>10 μM Nifedipine</td>
<td>27.01 ± 3.73</td>
<td>14.54 ± 2.29</td>
</tr>
<tr>
<td>100 μM Nifedipine</td>
<td>25.33 ± 3.37</td>
<td>12.27 ± 1.70</td>
</tr>
</tbody>
</table>

Table 2b
Effect of Y-27632 (20 min incubation for each concentration) on the contractile response of the human cavernosal strips to phenylephrine (Phe, 1 μM) or KCl (80 mM).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Responses to Phe</th>
<th>Responses to KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>93.9 ± 2.55</td>
<td>88.20 ± 3.88</td>
</tr>
<tr>
<td>0.3 μM Y27632</td>
<td>56.92 ± 3.91</td>
<td>52.21 ± 2.06</td>
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<tr>
<td>3 μM Y27632</td>
<td>24.73 ± 4.43</td>
<td>26.11 ± 3.46</td>
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<tr>
<td>30 μM Y27632</td>
<td>13.90 ± 2.35</td>
<td>15.45 ± 2.70</td>
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</tbody>
</table>
4. Discussion

The present study has demonstrated that PMF has a relaxant activity on isolated human cavernosum, a key process for producing a penile erection (Prieto, 2008; Wagner et al., 1989). Mechanisms responsible for the relaxation were explored to determine if PMF acts (1) as a nitric oxide stimulator or a guanylate cyclase stimulator, as an inhibitor of phosphodiesterase activity or by opening of K⁺ channels, (2) by blocking of voltage-dependent calcium channels, (3) as a Rho-kinase inhibitor, (4) as a store-operated calcium channel inhibitor, or (5) by inhibition of intracellular calcium mobilization.

Table 2c
Effect of 3, 5, 7, 3'-4'-pentamethoxyflavone (PMF, 20 min incubation for each concentration) on the contractile response of the human cavernosal strips to phenylephrine (Phe, 1 μM) or KCl (80 mM).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Slope of phasic contraction (% contraction/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Responses to Phe</td>
</tr>
<tr>
<td>Control</td>
<td>91.93 ± 3.58</td>
</tr>
<tr>
<td>0.01 mM PMF</td>
<td>71.60 ± 7.4</td>
</tr>
<tr>
<td>0.03 mM PMF</td>
<td>29.38 ± 6.94</td>
</tr>
<tr>
<td>0.1 mM PMF</td>
<td>3.73 ± 0.98</td>
</tr>
<tr>
<td>0.3 mM PMF</td>
<td>3.48 ± 2.07</td>
</tr>
<tr>
<td>1.0 mM PMF</td>
<td>Not studied</td>
</tr>
</tbody>
</table>

The slopes of the phasic contraction produced by phenylephrine (1 μM) and KCl (80 mM) were calculated from the linear portion of the curve (0–1 min) using a regression line analysis computer program (Microsoft Office Exel 2007). Data represent a mean ± S.E.M of 6 human cavernosal strips each obtained from a different person.

Table 3
Contractile response of the human cavernosum to phenylephrine (Phe, 1 μM, 100%), or to thapsigargin (TG, 3 μM, relative to Phe) and the percentage inhibitory effect of Y-27632 (Y, 30 μM), SKF-96365 (SKF, 100 μM) or 3, 5, 7, 3' 4'-pentamethoxyflavone (PMF, 0.3 mM) on human cavernosum precontracted with thapsigargin in the normal Krebs solution in the absence (left) or presence (right) of nifedipine (Nife).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Contraction</th>
<th>Treatment</th>
<th>% Contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe (18)</td>
<td>100</td>
<td>Phe (18)</td>
<td>100</td>
</tr>
<tr>
<td>TG (control, 18)</td>
<td>100.57 ± 4.13</td>
<td>TG (control, 18)</td>
<td>106.76 ± 4.87</td>
</tr>
<tr>
<td>TG + Y-27632 (6)</td>
<td>1.84 ± 1.2a</td>
<td>TG + Nife (18)</td>
<td>32.06 ± 3.80a</td>
</tr>
<tr>
<td>TG + SKF-96365 (6)</td>
<td>0.00 ± 0.0a</td>
<td>TG + Nife + SKF-96365 (6)</td>
<td>0.00 ± 0.0a</td>
</tr>
<tr>
<td>TG + PMF (6)</td>
<td>1.63 ± 0.62b</td>
<td>TG + Nife + PMF (6)</td>
<td>2.37 ± 1.58b</td>
</tr>
</tbody>
</table>

Data represents a mean ± S.E.M. Numbers in parentheses show the number of human cavernosal strips each obtained from a different person. *Significantly lower than control group, P < 0.05. **Significantly lower than the one with TG+Nife, P < 0.05.

Fig. 5. Effects of Y-27632 (Y, 30 μM), SKF-96365 (SKF, 100 μM) or 3, 5, 7, 3', 4'-pentamethoxyflavone (PMF, 0.3 mM) on the contractile responses of the human cavernosum to phenylephrine in normal Krebs's (left) or in Ca²⁺-free Kreb's solution (right). Each point represents a mean ± S.E.M of 6 human cavernosal strips each from a different person (n=6). *Significantly lower than the control (○) group, P < 0.05.
Our finding that LNA inhibited the relaxant activity of the PMF only at a low concentration suggests that PMF has only a weak effect on the stimulation of nitric oxide release. Furthermore, the finding that ODQ, TEA or glybenclamide did not inhibit the relaxant activity of the PMF indicates that PMF probably does not act as a stimulator of soluble guanylate cyclase, does not open potassium channels, it probably also affects other pathways.

Although PMF might inhibit the voltage-dependent Ca\(^{2+}\) channel, it probably also affects other pathways. PMF might act as a store-operated Ca\(^{2+}\) channel inhibitor, and further experiments were carried out in the presence of thapsigargin, a specific sarcoplasmic-endoplasmic reticulum Ca-ATPase (SERCA) pump inhibitor (Quinn et al., 2004, 2006; Thastrup et al., 1990), to deplete the intracellular Ca\(^{2+}\) store, which then would stimulate the opening of the plasma membrane store-operated Ca\(^{2+}\) channels to add to a refilling of the intracellular stores (Putney, 2011; Parekh and Putney, 2005). In the normal Krebs solution, incubation of the human cavernosal strip with thapsigargin caused a slow increase in human cavernosal strip contraction that reached its maximal contraction (20 min) to the same extent as that produced by phenylephrine (5 min); this result is similar to that reported by Takemura et al. (1991). In Ca\(^{2+}\)-free medium no response to thapsigargin was obtained. In the presence of nifedipine, PMF had an inhibitory effect on the Ca\(^{2+}\)-free phenylephrine C-R curve, and PMF and SKF-96365 caused a further inhibition the phenylephrine C-R curve (see Fig. 7F). These results suggest that PMF does not act as a store-operated Ca\(^{2+}\) channel inhibitor.

PMF and Y-27632, a Rho-kinase inhibitor (Ishizaki et al., 2000; Uehata et al., 1997), depressed the tonic contraction and slope of phasic contraction produced by phenylephrine and KCl. In Ca\(^{2+}\)-free solution, PMF caused a relaxation of about 50%, while Y-27632 caused complete relaxation (Fig. 7). Thus, it is unlikely that PMF acts as an inhibitor of the Rho-kinase. This was confirmed when it was found that PMF caused a depression of the C-R curves of the human cavernosal strips to phenylephrine to the same extent in both the normal Krebs solution with nifedipine and in the Ca\(^{2+}\)-free Krebs solution (see Fig. 5E and F), whereas Y-27632 inhibited the C-R curve in the normal Krebs solution and completely suppressed the phenylephrine C-R curve in the Ca\(^{2+}\)-free Krebs solution. These results indicated that it is unlikely that PMF plays any role as a Rho-kinase inhibitor.

In vascular smooth muscle, the \(\alpha\)-adrenoceptor-induced contraction in Ca\(^{2+}\)-free medium is initiated by Ca\(^{2+}\) release from the intracellular (IP3) store (Cortes et al., 1997; Takemura et al., 1991). That PMF antagonized the phenylephrine-induced contraction of the human cavernosal strips suggests that PMF may play a role as a Ca\(^{2+}\) channel inhibitor. This was additionally supported by our finding that sildenafil but not PMF caused a similar inhibitory effect to that of nifedipine on the phenylephrine-induced human cavernosal strip contraction, the maximum inhibition elicited by the PMF is about 2-fold greater than that of the nifedipine. Taken together these findings suggest that although PMF might inhibit the voltage-dependent Ca\(^{2+}\) channels, it probably also affects other pathways.

In vascular smooth muscle, the \(\alpha\)-adrenoceptor agonist, phenylephrine, induces an initial phasic contraction followed by a tonic contraction. The initial contraction is mediated by the release of intracellular Ca\(^{2+}\) from the sarcoplasmic reticulum (Noguera et al., 1998) whereas the sustained tonic contraction results from Ca\(^{2+}\) influx via the voltage-dependent Ca\(^{2+}\) channels (Abebe et al., 1990; Akata, 2007; Nelson et al., 1988). Our finding that PMF antagonized the phenylephrine-induced contraction of the human cavernosal strips suggests that PMF may play a role as a Ca\(^{2+}\) channel inhibitor. This was additionally supported by our finding that PMF further attenuated the reduction in the contraction response to phenylephrine caused by nifedipine. Furthermore, it was found that PMF caused a concentration-dependent depression of the slope of the phasic contraction induced by KCl, a depolarizing agent, likely dependent on the rate of influx of extracellular Ca\(^{2+}\) via the L-type Ca\(^{2+}\) channels. Nifedipine had similar effects on KCl contractions. However, although PMF showed a similar inhibitory effect to that of nifedipine on the phenylephrine induced human cavernosal strip contraction, the maximum inhibition elicited by the PMF is about 2-fold greater than that of the nifedipine. Taken together these findings suggest that although PMF might inhibit the voltage-dependent Ca\(^{2+}\) channels, it probably also affects other pathways.

5. Conclusion
PMF exerted a relaxant activity on phenylephrine precontracted isolated human cavernosum. The results indicate that PMF may act as an L-type Ca\(^{2+}\) channel inhibitor and also as an inhibitor of the intracellular Ca\(^{2+}\) mobilization from the sarcoplasmic reticulum. PMF had only a weak effect on the release of nitric oxide, and it is unlikely that PMF acts as a soluble guanylate cyclase stimulator, a Ca\(^{2+}\) sensitive K\(^+\) channel opener, an ATP

![Fig. 6. Contractile responses of the human cavernosum to phenylephrine in Ca\(^{2+}\)-free medium before and after blocking the SERCA pump inhibitor for 40 min with thapsigargin (3 μM). Each point represents a mean ± S.E.M. of 6 human cavernosal strips each from a different person (n=6). *Significantly lower than the control (C) group, P<0.05.](image-url)
Fig. 7. Effects of Y-27632 (Y, 30 mM), SKF-96365 (SKF, 100 mM), nifedipine (3 mM) and/or 3, 5, 7, 3', 4'-pentamethoxyflavone (PMF, 0.3 mM) on the phenylephrine induced contractile responses of human cavernosum, pretreated with thapsigargin (3 mM) to depleted Ca^{2+} in the sarcoplasmic reticulum, in Ca^{2+}-free Krebs solution. Each point represents a mean ± S.E.M. of 6 human cavernosal strips each from a different person (n = 6). *Significantly lower than the control (○) group, P < 0.05. †Significantly lower than the one with SKF (●), SKF + Nife (■), PMF (▲) or Nife + PMF (■), P < 0.05.

PMF inhibited L-type Ca^{2+} channel and mobilization of Ca^{2+} from sarcoplasmic reticulum (SR), and might (?) stimulate release of Ca^{2+} from other intracellular stores. PMF did not appear to affect (X) Ca-activated K-channels (Kca), ATP-activated K-channels (KATP), store-operated channels (STOC), or Rho-kinase.

Fig. 8. The proposed mechanisms that would be responsible for the relaxant activity of 3, 5, 7, 3', 4'-pentamethoxyflavone (PMF) on the human cavernosum.
sensitive K⁺ channel opener, a Rho-kinase inhibitor or a store-operated Ca2⁺ channel inhibitor. On the other hand, results also showed that PMF may stimulate the release of Ca2⁺ from other minor intracellular Ca2⁺ stores. Taken together, these studies indicate that PMF causes relaxation of the human cavernous primarily through mechanisms associated with voltage-dependent Ca2⁺ channels and calcium mobilization (Fig. 8).

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References

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