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Vascular Effects of Different Lipophilic Components of "Danshen", a Traditional Chinese Medicine, in the Isolated Porcine Coronary Artery

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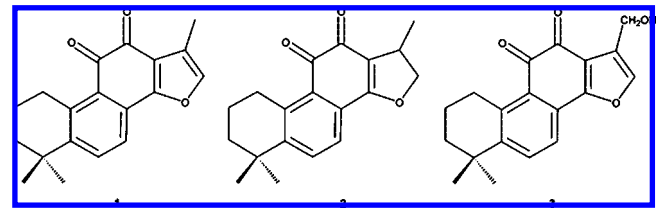
"Danshen" has been used for the treatment of various cardiovascular diseases in the People's Republic of China for many years. Two different forms of "Danshen" exist, with the roots of *Salvia miltiorrhiza* being the traditional form and the roots of *Salvia przewalskii* being a surrogate used in the western areas of mainland China. The most abundant lipophilic diterpene quinones present in *S. miltiorrhiza* and *S. przewalskii* roots, tanshinone IIA (**1**) and cryptotanshinone (**2**), inhibited contraction of the isolated porcine coronary artery to the thromboxane A_2 analogue, U46619. Przewaquinone A (**3**), a lipophilic diterpene quinone present only in *S. przewalskii*, induced a similar but greater inhibitory action on vascular contraction than **1** and **2**. This effect of **3** was endothelium-independent and reversible. The present results suggest that **3** is more potent than **1** and **2** and may contribute to a great extent to the ability of *S. przewalskii* roots to inhibit vascular contractions.

"Radix Salviae Miltiorrhiza", also known as "Danshen", is the dried root and rhizome of *Salvia miltiorrhiza* Bunge (Labiatae). It is a traditional Chinese medicine that has been used for the treatment of various cardiovascular diseases.[1,2] This action of *S. miltiorrhiza* roots may be related to its ability to increase coronary blood flow and improve heart function, as demonstrated in isolated heart preparations.[2−4] As *S. miltiorrhiza* is cultivated mainly in northeastern mainland China, another species of the *Salvia* genus, *Salvia przewalskii* Maxim, is used as a surrogate for *S. miltiorrhiza* in western areas of mainland China, such as Gansu and Yunnan Provinces, due to its wide distribution in these regions.[5−7] The dried root and rhizome of *S. przewalskii* is commonly known as "Gansu danshen".

While botanical classification suggests the potential of *S. przewalskii* to be a surrogate of *S. miltiorrhiza*, scientific evidence for similar biological activities between these two species of the *Salvia* genus is limited. Comparison of the chemical composition of *S. miltiorrhiza* and *S. przewalskii* roots indicates that both species contain large amounts of the bioactive diterpene quinones tanshinone IIA (**1**) and cryptotanshinone (**2**), whereas the related compound przewaquinone A (**3**) is present only in *S. przewalskii*.[6−8] Therefore, *S. przewalskii* may possess biological actions that are both similar to and distinct from *S. miltiorrhiza* roots. Since the efficacy of *Salvia* species is correlated to their relative content of bioactive component(s),[9] variations in the amounts of **1** and **2** between *S. przewalskii* and *S. miltiorrhiza*[7,10] may lead to the differential effectiveness of these two *Salvia* species in treating cardiovascular diseases. As a result, in the present study, we have attempted to determine whether the actions of *S. miltiorrhiza* in the cardiovascular system may be attributed to its lipophilic components, **1** and **2**, using an isolated coronary arterial model. Moreover, the lipophilic compound that is restricted to *S. przewalskii*, **3**, was examined for its potential role in mediating a cardiovascular protective action of *S. przewalskii*.

Results and Discussion

According to the Pharmacopoeia of the People's Republic of China,[11] *S. miltiorrhiza* roots exert a beneficial action in the cardiovascular system, resulting in the elimination of blood stasis and the enhancement of blood flow. An in vitro study with isolated coronary arteries indicated that *S. miltiorrhiza* roots act as a vasodilating agent, and this effect is not species-dependent.[12] Using a commercially available form of *S. miltiorrhiza*, Lam et al. showed that the lipophilic fraction relaxed the phenylephrine-contracted rat femoral artery only at concentrations ≥20 μg/mL.[13] In the present study, we have demonstrated that the lipophilic fraction of *S. miltiorrhiza* roots induced complete relaxation in porcine coronary arteries that were contracted with U46619 (30 nM) at a concentration of 0.1 mg/mL (Figure 1). At lower concentrations, the lipophilic fraction reduced arterial responses in a dose-dependent manner to the contracting agent, U46619 (Figure 2). At a concentration of 10 μg/mL, the lipophilic fraction also did not cause any significant relaxation in the porcine coronary artery, as the maximum contraction to U46619 was not affected significantly (Figure 2). Nevertheless, it was effective in reducing the sensitivity of the porcine coronary artery to contraction, as indicated by a shift to the right of the concentration−contraction curve to U46619 (Figure 2), with a significant reduction in the log ED_{50} values of U46619 from −8.5 ± 0.07 to −8.1 ± 0.14 in the absence and presence of the lipophilic fraction (10 μg/mL), respectively.



As the lipophilic fraction consisted of a large amount of diterpene quinones, these compounds may be responsible for the vasodilatory action of *S. miltiorrhiza* roots. Studies with the major diterpene quinones, **1** and **2**, indicated that both compounds were effective in inhibiting vascular contraction to U46619 (Figure 3), whereas the vehicle, PVP, did not affect U46619-induced contraction. Between them, the inhibitory action of **2** appears to be more potent than **1**, since at the concentration tested (50 μg/mL), both the maximum contraction and the ED_{50} value of U46619 were affected in the presence of **2**, whereas **1** only shifted the concentration−contraction curve of U46619 to the right (Table 1 and Figure 3).

While both **1** and **2** mimicked the lipophilic fraction of *S. miltiorrhiza* roots in inhibiting vascular contraction, neither was

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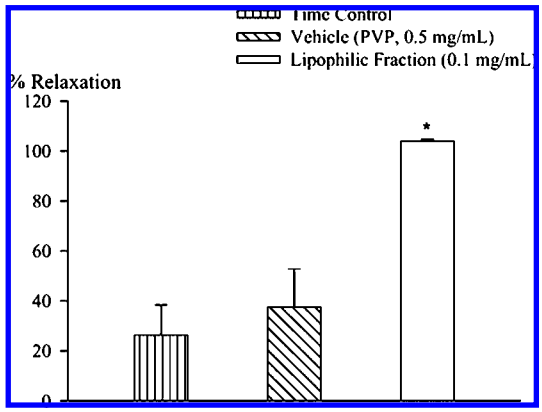

Figure 1. Maximum relaxation induced by the lipophilic fraction of *S. miltiorrhiza* roots or their vehicle, polyvinylpyrrolidone K30 (PVP), in the isolated porcine coronary artery with an endothelium. Relaxation was expressed as percentage change from the contracting level induced by U46619 (30 nM). Data are represented as mean ± standard error of the mean for five individual experiments. [* denotes significant difference ($p < 0.05$) compared with the control group].

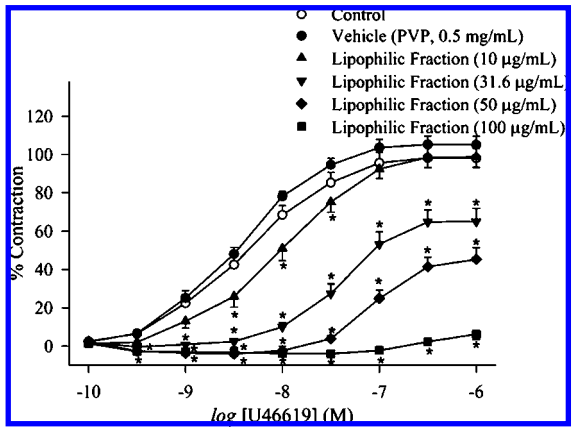

Figure 2. Contractions by increasing concentrations of U46619 in the presence of the lipophilic fraction of *S. miltiorrhiza* roots or its vehicle, polyvinylpyrrolidone K30 (PVP), in the isolated porcine coronary artery with an endothelium. Each contraction was expressed as a percentage of the average values of the two contracting levels induced by potassium chloride (30 mM). Data are represented as mean ± standard error of the mean for 6−7 individual experiments [* denotes significant difference ($p < 0.05$) compared with the control group].

as effective as the lipophilic fraction at the concentration tested (50 μg/mL). This observation, therefore, suggested that, in addition to **1** and **2**, *S. miltiorrhiza* roots contain other lipophilic components with inhibitory effects on vasoconstriction. Alternatively, there may be an additive or even synergistic effect of different components of *S. miltiorrhiza* roots on the vasculature. Other diterpene quinones, such as tanshinone I and dihydrotanshinone I, have been isolated in *S. miltiorrhiza* roots, although they are present in smaller quantities than **1** and **2**.[14] Mixtures of tanshinones were suggested to be equally effective to the aqueous extract of *S. miltiorrhiza* in relaxing isolated rabbit blood vessels.[12] On the other hand, compound **2**, isolated from a different source of crude *S. miltiorrhiza*, was found to be more potent than the lipophilic fraction from which it was extracted.[15] It should be noted that the composition of crude *S. miltiorrhiza* is known to vary significantly depending on the environment in which it is grown.[16] Variations in the content of different components of *S. miltiorrhiza* may also be the result of differences in extraction and processing methods.[16] Current practice has relied mainly on the determination of the content of **1**

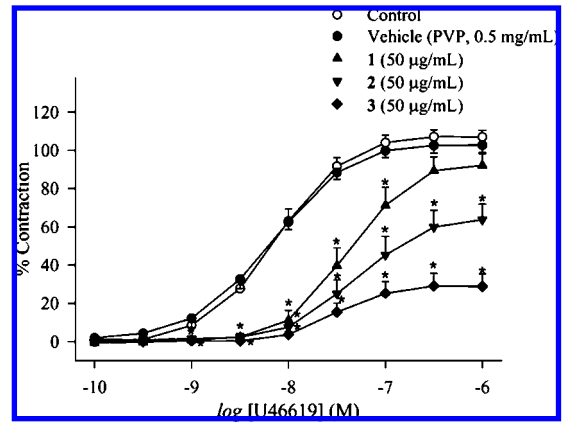

Figure 3. Contractions by increasing concentrations of U46619 in the presence of tanshinone IIA (**1**), cryptotanshinone (**2**), przewaquinone A (**3**), or their vehicle, polyvinylpyrrolidone K30 (PVP), in the isolated porcine coronary artery with an endothelium. Each contraction was expressed as a percentage of the average values of the two contracting levels induced by potassium chloride (30 mM). Data are represented as mean ± standard error of the mean for eight individual experiments [* denotes significant difference ($p < 0.05$) compared with the control group].

Table 1. ED_{50} Values and Maximum Contractions (E_{max}) of Concentration−Contraction Curves of U46619 in the Presence of **1**, **2**, **3**, or Their Vehicle (PVP) in the Porcine Coronary Artery[a]

treatment group	ED_{50}	E_{max}
vehicle	−8.3 ± 0.12	104.3 ± 4.14
1	−7.4 ± 0.10[b]	92.9 ± 6.59
2	−7.2 ± 0.11[b]	64.9 ± 7.93[b]
3	−7.4 ± 0.09[b]	29.2 ± 6.56[b]

[a] Data are represented as mean ± standard error of the mean for eight individual experiments. [b] Denotes significant difference ($p < 0.05$) compared with the vehicle group.

for the quality control of *S. miltiorrhiza*.[11] However, with regard to vascular activity, the present results clearly indicated that **1** plays only a minor role, thus arguing against the appropriateness of using it as a standard reference for the quality control of *S. miltiorrhiza* as a vasoactive compound.

Unlike *S. miltiorrhiza,* of which the biological activities have been extensively studied,[3,4,17−20] much less research has been conducted to elucidate the pharmacological actions of *S. przewalskii*, a species that is used commonly as a surrogate for *S. miltiorrhiza* in western areas of mainland China. Examination of the chemical composition of *S. miltiorrhiza* and *S. przewalskii* indicated that both contain high concentration levels of diterpene quinones, with the majority being **1** and **2**.[6−8] On the other hand, the diterpene quinone **3** is present only in *S. przewalskii* but not in *S. miltiorrhiza*.[6,21] In the present study, compound **3**, like the other diterpene quinones, **1** and **2**, significantly inhibited U46619-induced contractions at 50 μg/mL (Figure 3). Moreover, **3** was the most effective among the diterpene quinones tested, as inhibition of U46619-induced contractions by **3** was the greatest among compounds **1**−**3**. As such, the presence of **3**, together with a higher content of **1** and **2**,[7,10] suggests that *S. przewalskii* is likely to have a higher potency in inhibiting vascular contractions than *S. miltiorrhiza*.

The finding that inhibition of U46619-induced contractions by **3** was reversed following their removal from the coronary artery (Figure 4) suggests that this effect was not caused by alteration in the expression of signaling molecules involved in vascular contraction. Since the extent to which **3** inhibited U46619-induced contractions was similar in the porcine coronary artery both with an endothelium (Figure 3) and without an endothelium (Figure 5a), the endothelium did not play a role in the mechanism of action of

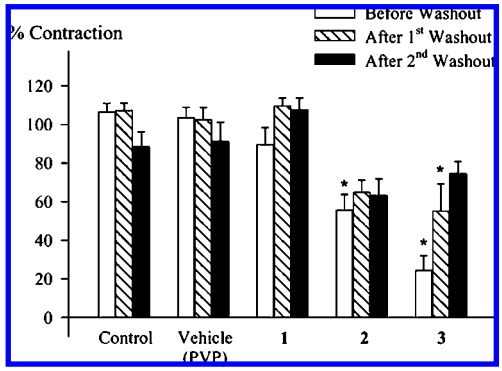

Figure 4. Contractions by U46619 (1 μM) in the presence or after the washout of tanshinone IIA (**1**), cryptotanshinone (**2**), przewaquinone A (**3**), or their vehicle, polyvinylpyrrolidone K30 (PVP), in the isolated porcine coronary artery with an endothelium. Each contraction was expressed as a percentage of the average values of the two contracting levels induced by potassium chloride (30 mM). Data are represented as mean ± standard error of the mean for six individual experiments [* denotes significant difference ($p < 0.05$) compared with the control group].

3. In addition to U46619, coronary arterial responses to another receptor-mediated agent, 5-hydroxytryptamine, and to a depolarizing agent, potassium chloride, were also inhibited in the presence of **3** (Figure 5). As such, the mechanism through which **3** exerted its inhibitory effect on vascular contractions is most likely downstream of receptor activation. While **3** was effective in inhibiting U46619- and 5-hydroxytryptamine-induced contractions at a concentration of 15 μg/mL, more than a 3-fold higher concentration (50 μg/mL) was required to significantly affect potassium chloride-induced contractions (Figure 5). This finding thus suggests that **3** inhibited vascular contraction through two different mechanisms. Inhibition of depolarization-mediated contractions may involve only one mechanism, most likely through inhibition of calcium influx or a vascular smooth muscle contractile mechanism. An additional mechanism, possibly through interfering with the G-protein coupling mechanism or inositol phosphate-induced calcium release from intracellular stores, may be responsible for the higher potency of **3** in inhibiting receptor-mediated contractions.

In conclusion, both forms of the "Danshen" crude drug, *S. miltiorrhiza* and *S. przewalskii*, possess vascular action, as they both contain components with inhibitory effects on vascular contractions. Among the three diterpene quinones tested, **3** was found to be the most effective, but it is present only in *S. przewalskii*. Together with the endothelium-independent nature of its action, **3** may be a suitable compound to use against vascular disorders, which are generally associated with endothelial dysfunction.

Experimental Section

General Experimental Procedures. Hearts from pigs (50−80 kg) of either sex were collected from a local slaughterhouse and were immersed immediately in cold modified Krebs-Henseleit solution (composition in mM: NaCl, 120; KCl, 4.76; $MgSO_4$, 1.18; $CaCl_2$, 1.25; $NaHCO_3$, 25; NaH_2PO_4, 1.18; glucose, 5.5). Left anterior descending and right coronary arteries were isolated. After removing fat and connective tissue, the arteries were cut into 3 mm ring segments. The arterial rings were then mounted between two stainless steel hooks in 5 mL of organ baths. One of these hooks was connected to a force transducer (model FT03, Grass Instrument Co., Quincy, MA) for measurement of isometric tension developed in the rings. In some experiments, the endothelium of the rings was removed by perfusing a solution of Triton X-100 (0.25% in modified Krebs-Henseleit solution) through the lumen of the arteries at a rate of 1 mL/min for 30 s. Arterial rings were maintained at 37 °C bubbled with a mixture of 95% oxygen and 5% carbon dioxide in Krebs-Henseleit solution. Before the start of the experiment, the rings were maintained at a resting tension of

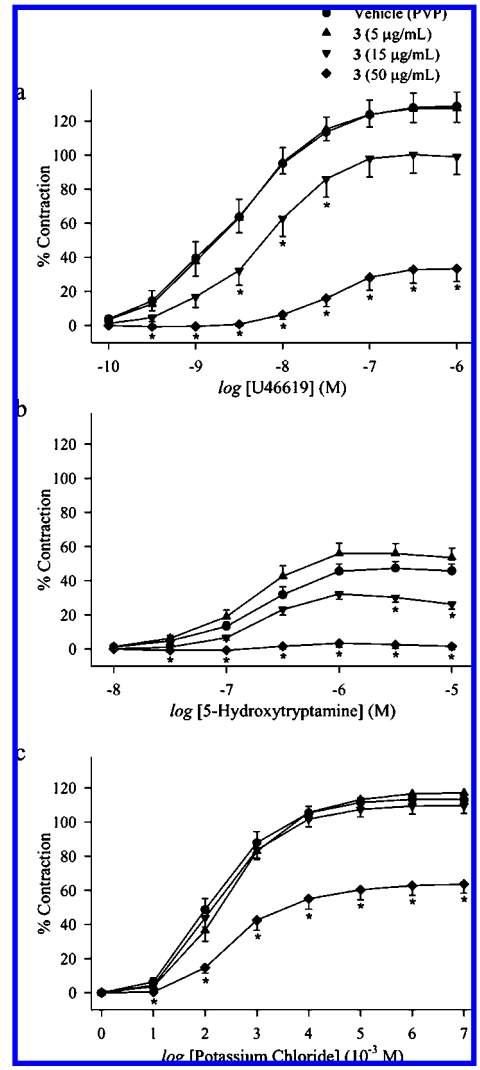

Figure 5. Contractions by increasing concentrations of (a) U46619, (b) 5-hydroxytryptamine, and (c) potassium chloride in the presence of przewaquinone A (**3**) or its vehicle, polyvinylpyrrolidone K30 (PVP), in the isolated porcine coronary artery without an endothelium. Each contraction was expressed as a percentage of the average values of the two contracting levels induced by potassium chloride (30 mM). Data are represented as mean ± standard error of the mean for 7−11 individual experiments [* denotes significant difference ($p < 0.05$) compared with the vehicle group].

2 g and equilibrated for 100 min with modified Krebs-Henseleit solution, which was changed regularly.

After the equilibration period, arterial rings were incubated with indomethacin (10 μM) for 20 min followed by contraction with the stable thromboxane A_2 analogue, U46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$; 30 nM). Once a plateau contraction was induced in arterial rings by U46619, bradykinin (1 μM) was then added to relax the arterial rings. In arterial preparations with an endothelium, rings with a contraction greater than 4 g and relaxation greater than 80% of the contracting level achieved by U46619 were considered viable. Viable rings were then incubated again with indomethacin (10 μM) for 20 min after the drug effects of U46619 and bradykinin were washed out. They were then contracted with U46619 (30 nM) again before being exposed to the lipophilic fraction of *S. miltiorrhiza* roots or the vehicle polyvinylpyrrolidone K30 (PVP).

For investigation of contracting responses, arterial rings were contracted with potassium chloride (30 mM) and relaxed with bradykinin (1 μM) after the equilibration period. This procedure was repeated once. For arterial rings with an endothelium, the sample tissues with an average contraction smaller than 4 g and an average relaxation smaller than 40% were discarded from the study. For arterial rings without an endothelium, tissues that developed an average contraction

smaller than 4 g and an average relaxation greater than 5% were not used. After the effects of potassium chloride and bradykinin were washed out with modified Krebs-Henseleit solution, the lipophilic fraction (10−100 μg/mL), or **1** (50 μg/mL), **2** (50 μg/mL), or **3** (5−50 μg/mL) or their vehicle, polyvinylpyrrolidone K30 (PVP, 0.5 mg/mL), were added into the organ bath. After incubation of these compounds with the arterial rings for 30 min, contractions were produced by a stepwise addition of U46619 (0.1 nM to 1 μM), 5-hydroxytryptamine (5-HT; 10 nM to 10 μM), or potassium chloride (10−70 mM). In some experiments, contraction was induced by U46619 (1 μM) after incubation of the tested compounds. After reaching the plateau contracting level, the effect of drugs was washed out by changing the modified Krebs-Henseleit solution, and arterial rings were then contracted again with U46619 (1 μM) to investigate the reversibility of drug effect.

Preparation of Lipophilic Extract of *S. miltiorrhiza* and Purification of Compounds 1−3. The roots of *S. miltiorrhiza* and *S. przewalskii* were collected from Jinzhai, Anhui Province, and Jiangyou, Sicuan Province, People's Republic of China, respectively, in October 2000, and were identified by one of the authors (D.-Y.Z.). A lipophilic fraction and compounds **1** and **2** were obtained from *S. miltiorrhiza*, whereas compound **3** was isolated from *S. przewalskii*. Briefly, *S. miltiorrhiza* roots (2 kg) were ground to a powder and extracted with cold diethyl ether (3 × 10 L). The resulting extract is termed the lipophilic fraction (3.2% w/w). This lipophilic fraction (60 g) was then further treated with sodium carbonate solution (5%) to give a neutral diethyl ether fraction (32 g). Compounds **1** (1.2 g) and **2** (0.14 g) were isolated from this neutral diethyl ether fraction using silica gel column chromatography with 10:1 (v/v) and 15:1 (v/v) petroleum ether−EtOAc, respectively, for elution. Compound **3** (0.075 g), on the other hand, was isolated from dried and powdered *S. przewalskii* roots (5 kg) by extraction with ethanol (95%, 3 × 25 L) followed by purification using silica gel column chromatography with 10:1 (v/v) petroleum ether−EtOAc for elution. The degree of purity of **1**−**3** was more than 98% as detected by HPLC analysis (column: Zorbax Eclipe XDB-C_{18}, 250 × 4.6 mm, 5 μm; mobile: MeOH−H_2O, 75:25; rate: 1 mL/min; temperature: 25 °C; UV: 270 nm). The isolated lipophilic fraction of *S. miltiorrhiza* and the pure compounds formed complexes with the vehicle polyvinylpyrrolidone K30 (PVP) for use in the pharmacological experiments. The ratio of the lipophilic fraction or pure compounds to PVP was 1 to 10 by mass.

Tanshinone IIA (1): mauve needles (methanol), mp 209−211 °C,[6] and spectroscopic data (^{1}H NMR, ^{13}C NMR, EIMS) consistent with literature values.[22]

Cryptotanshinone (2): orange-red needles, mp 190−191.5 °C, $[\alpha]^{21}_D$ −78,[6] and spectroscopic data (^{1}H NMR, ^{13}C NMR, EIMS) consistent with literature values.[23,24]

Przewaquinone A (3): orange-red prisms, mp 171−173 °C,[6] and spectroscopic data (^{1}H NMR, ^{13}C NMR, EIMS) consistent with literature values.[22]

Chemicals. NaCl, $NaHCO_3$, $NaH_2PO_4 \cdot 2H_2O$, KCl, and D(+)-glucose were purchased from BDH Laboratory Supplies, Poole, UK. $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, bradykinin acetate, and 5-hydroxytryptaimine hydrochloride were obtained from Sigma Chemicals Co., St. Louis, MO. U46619 was purchased from Biomol, Plymouth Meeting, PA. Triton X-100 was obtained from Pharmacia Biotech, Uppsala, Sweden. A stock solution of U46619 was dissolved in ethanol, with the final concentration of ethanol in each bath being ≤0.1%. Indomethacin (1 mM) was dissolved in sodium carbonate solution (1 mM). All other drugs used were dissolved in distilled water.

Statistical Analysis. All results were expressed as mean ± standard error of the mean of individual experiments, each of which was conducted with arterial rings that were isolated from different porcine hearts. For relaxation experiments, the degree of relaxation induced by bradykinin or different test compounds was expressed as a percentage of the contracting level induced by U46619 (30 nM) or potassium chloride (30 mM). Vascular contraction was calculated as a percentage of the average of the two contractions that were induced by potassium chloride (30 mM) before incubation of the tested compounds. Analysis of variance (ANOVA) and Dunnett's test were applied to analyze the significance of the mean differences between groups. The computer statistical package used was SPSS (SPSS Inc., Chicago, IL), and statistical significance was determined by a p value ≤ 0.05.

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