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Vascular myorelaxing activity of isolates from South African Hyacinthaceae partly mediated by activation of soluble guanylyl cyclase in rat aortic ring preparations

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Abstract

The vasorelaxing effect of isolates (compounds **1, 2, 3**, and **4** (homoisoflavanones), compound **5** (sesquiterpenoid), compounds **6** and **7** (bufadienolides)) from the South African Hyacinthaceae has been assessed using rat aortic ring preparations. Compounds **2, 3**, and **4** inhibited the tonic contraction induced by both 60 mM K^+ (K60) and phenylephrine, compound **3** being the most potent. Compounds **5, 6**, and **7** caused a modest concentration-dependent relaxation, whereas compound **1** was ineffective. Under K25- or K60-induced depolarization, compound **3** displayed antispasmodic effects not reversed by tetraethylammonium. Under precontraction induced with phenylephrine, compound **3** shifted to the left the concentration–relaxation curves of either isoprenaline or sodium nitroprusside. 1 H-[1,2,4] oxidazolol [4,3-a] quinoxalin-1-one shifted to the right the concentration–relaxation curve of compound **3**, while 3′-isobutyl-1-methylxanthine had no effect. In the absence of extracellular Ca^{2+}, compound **3** (estimated pIC50 = 4.66) and ryanodine reduced the response to phenylephrine. Phenylephrine-stimulated influx of extracellular Ca^{2+} was markedly reduced when tissues were pretreated with compound **3** (pIC50 = 5.14) or nifedipine, but stimulated by ryanodine. Compound **3** partially antagonized the contraction induced by phorbol 12-myristate-13-acetate. To our knowledge, this has been the first account describing the vasodilating activity of homoisoflavonoids: compound **3** proved an effective vasorelaxing agent, partly acting via the activation of soluble guanylyl cyclase.

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Introduction

Southern Africa is one of two world centres of diversity in the Hyacinthaceae, harbouring more than one third of the world's family representatives within three of the five subfamilies (Speta 1998). Whereas the activity of bufadienolides isolated from representatives of the subfamily Urgineoideae has historically attracted the attention of toxicologists (Pohl et al 2001), pharmacological research has more recently focussed on the homoisoflavonoids typically sourced from members of the subfamily Hyacinthoideae. Along the highly populated eastern seaboard region of South Africa, this subfamily is particularly popular in ethnomedicine (Von Ahlefeldt et al 2003).

The Southern Sotho treat piles (haemorrhoids present following varicose dilation of veins of the superior or inferior haemorrhoidal plexus) with preparations of *Eucomis autumnalis* (Mill.) Chitt. (Maliehe 1997). Several regional tribes treat headaches and hangovers using this same species (Watt & Breyer-Brandwijk 1962; Felhaber 1997). Furthermore, Mander et al (1995) reported that *E. autumnalis* bulb preparations were applied as an enema to improve sexual prowess (*uqundu wenkunzi*) in men. Several of the prominent ethnomedicinal genera such as *Eucomis* L'Hér. have recently received attention from pharmacologists (see Koorbanally et al (2006b) and references within). Accordingly, anti-inflammatory activity has been demonstrated for homoisoflavanones (Della Loggia et al 1989), of which several such compounds have been isolated in quantity from *E. autumnalis* (Pohl et al 2001). Consequently, traditional usage of these pain- and inflammation-relieving herbal preparations has been considered previously, validated through inhibition of cyclooxygenase enzymes (COX-1 and COX-2) (Taylor & Van Staden 2002).

The vasoactivity of isolates from the Hyacinthaceae (particularly the Hyacinthoideae) has been considered as a working hypothesis in view of the traditional use of family members for treating ailments associated with blood vessel dysfunction. Accordingly, the aim of this work was to assess seven compounds (**1–7**; Figure 1) from South African hyacinthacs for their in-vitro effects on the vascular system, by means of rat aortic ring preparations. The homoisoflavonoid (*R*)-3′,5-dihydroxy-4′,7-dimethoxyspiro [2H-1-benzopyran-3(4H),7′-bicyclo[4.2.0]-octa[1,3,5]-trien]-4-one (compound **3**), was the most powerful and promising vasorelaxing agent: the mechanism underlying this effect was investigated.

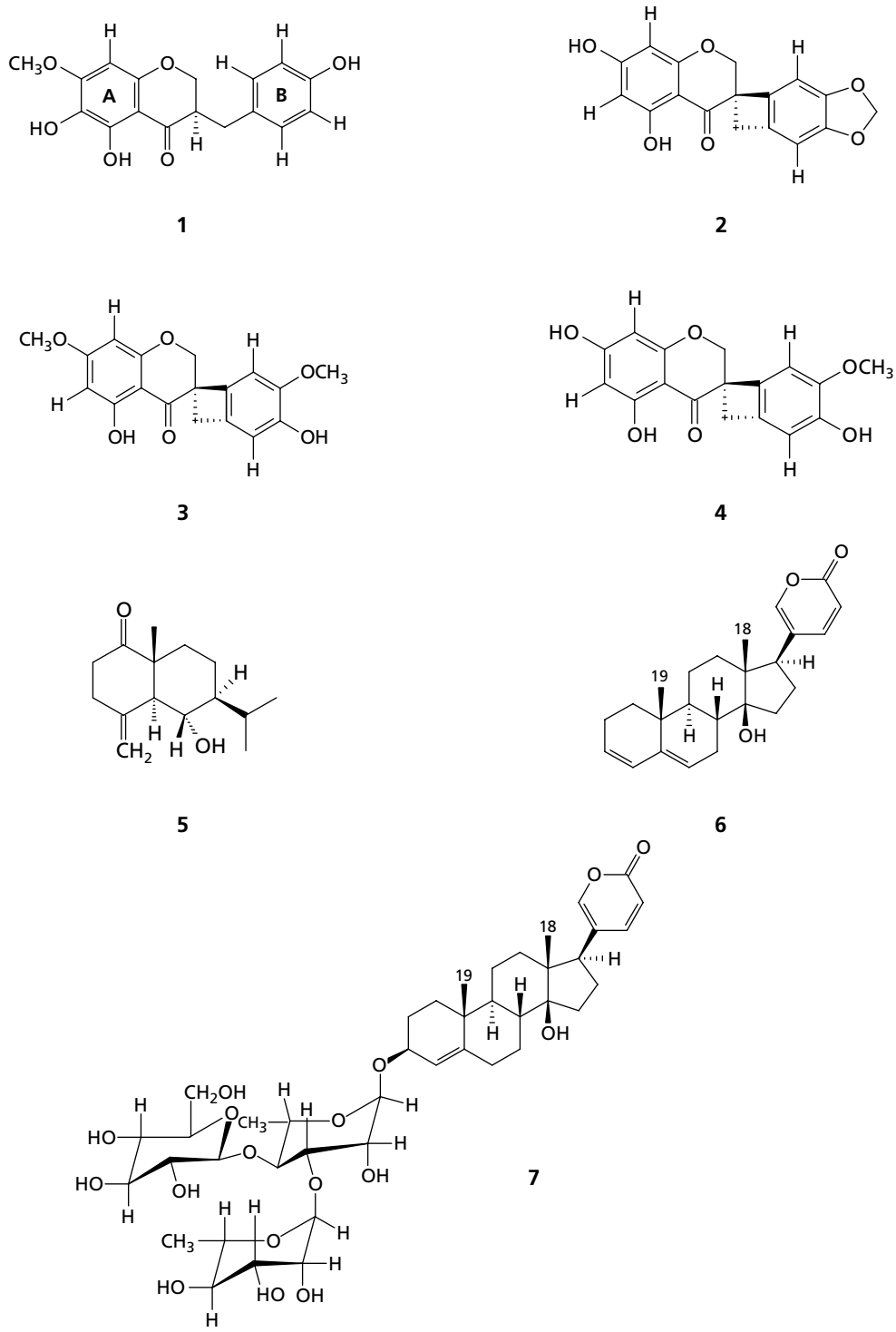

Figure 1 Chemical structures of compounds **1–7**.

Materials and methods

Extraction and isolation

Bulbs of *Drimiopsis maculata* Lindl., *Eucomis schiffii* Reyneke (Hyacinthoideae), *Urginea epigea* R.A.Dyer and *Drimia altissima* (L.f.) Ker Gawl. (Urgineoideae) of the Hyacinthaceae family were air-dried and macerated. The bulbs were then extracted on a Labcon Mechanical shaker (Labdesign Engineering (Pty) Ltd Maraisburg, South Africa), using $MeCl_2$ and MeOH for 48 h each, respectively. Each extract was separated using column chromatography over silica gel (Merck 9385; Darmstadt, Germany) with an increasing polarity solvent gradient (100% $MeCl_2$ →100% MeOH). Repeated column chromatography of the extracts afforded the pure compounds **1–7**. Structural elucidation was carried out using mass spectrometry and 2D NMR techniques. NMR spectra were recorded in $CDCl_3$ on a 400 MHz Varian spectrometer (Darmstadt, Germany) and mass spectra were obtained on a Kratos high-resolution MS 9/50 spectrometer (Shimadzu, Japan). The exact conditions used for each chromatographic separation and physical data for compounds isolated are given elsewhere (Koorbanally et al 2001, 2004, 2006a, c; Pohl et al 2000).

Aortic ring preparation

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animal protocols used were reviewed and approved by the Animal Care and Ethics Committee of the Università degli Studi di Siena, Italy (31-1-2006). Aortic rings (1.5/2.5-mm wide), either endothelium-intact or –denuded, were prepared from male Sprague–Dawley rats (347.2 ± 8.5 g, n = 46; Charles River Italia, Calco, Italy) anaesthetized with a mixture of Ketavet (30 mg kg^{-1}; Gellini, Italy) and Rompum (8 mg kg^{-1}; Bayer, Germany), decapitated and bled, as described by Fusi et al (2000).

Equilibration of rings was performed as described by Fusi et al (2000). The presence of functional endothelium was assessed in all preparations by testing the capacity of acetylcholine (10 μM) to induce a relaxation ≥75% of the phenylephrine-induced contraction; on the contrary, a relaxation < 10% was considered representative of the lack of the endothelial layer. Under these conditions, maximal plateau values for active tension of either 307.7 ± 10.2 mg (n = 198, endothelium-denuded) or 219.0 ± 27.5 mg (n = 25, endothelium-intact; $P < 0.01$, Student's *t*-test for unpaired samples) were obtained. Experiments were mostly conducted on endothelium-denuded rings unless otherwise indicated. Control preparations were treated with the drug vehicle only.

Relaxing effect of compounds 1–7 on aortic rings contracted with K^+ or phenylephrine

This series of experiments was carried out to screen compounds **1–7** and select the most active compounds. Steady tension was evoked either by 60 mM K^+ (K60) or by 0.3 μM phenylephrine and then the drugs under investigation were added cumulatively (0.01–100 μM). Relaxation was then evaluated as a percentage of the initial response to either K60 or phenylephrine, taken as 100%. Only the isolates relaxing the induced tone by more than 50% were evaluated on endothelium-intact rings precontracted with 0.3 μM phenylephrine, to select the most active compound and to characterize its mechanism of action.

Assessment of K^+-channel opening activity

Vasorelaxation induced by K^+-channel openers is inversely correlated to the extracellular concentration of K^+. Therefore, the antispasmodic activity of the most active compound was evaluated in rings under different conditions of high K^+-evoked depolarization (25 or 60 mM K^+, respectively) either in the absence or in the presence of 10 mM tetraethylammonium. Responses were then evaluated as a percentage of the initial response to K60, taken as 100%.

Effect on Ca^{2+} release from intracellular stores and on extracellular Ca^{2+} influx triggered by phenylephrine

Vasorelaxing agents can act by interfering either with Ca^{2+} release from intracellular stores and/or with Ca^{2+} influx, both triggered by phenylephrine. Therefore, to assess the mode of action of the most active compound, physiological saline solution (PSS) was replaced with a Ca^{2+}-free solution containing 1 mM EGTA. Rings were exposed to this solution for 5 min (David et al 2002) and then stimulated with 10 μM phenylephrine (this response was taken as an index of the internal stored Ca^{2+}). External Ca^{2+} was then restored in the presence of phenylephrine and the ensuing contraction was taken as an index of the influx of Ca^{2+} from the extracellular space triggered, in part, by the emptied stores, and, in part, by α_1-adrenoceptor stimulation. Contractions were obtained after a 30-min incubation period with vehicle (dimethyl sulfoxide or ethanol), the most active compound (3–100 μM), ryanodine (30 μM) or nifedipine (300 nM). Responses were evaluated as percentage of the contraction induced by 0.3 μM phenylephrine in PSS taken as 100%.

Effect on relaxation induced by isoprenaline or sodium nitroprusside

Compounds capable of augmenting intracellular cyclic nucleotide levels increase the potency of vasorelaxing drugs acting via these pathways. Therefore, the most active compound (3 or 10 μM) or vehicle was left in contact with rings for 30 min in PSS, while tone was induced by the addition of 0.3 μM phenylephrine; a concentration–response curve for either isoprenaline or sodium nitroprusside was constructed. Relaxations were then evaluated as a percentage of the initial response to phenylephrine, taken as 100%.

Effect of 3′-isobutyl-1-methylxanthine (IBMX) or 1 H-[1,2,4] oxidazolol [4,3-a] quinoxalin-1-one (ODQ) on relaxation induced by the most active compound

Intracellular cyclic nucleotide concentration increases as a result of either cyclase enzyme stimulation or phosphodiesterase inhibition. Therefore, after tone induction with 0.3 μM phenylephrine, the non-specific phosphodiesterase inhibitor IBMX (0.1–1 μM) was added. This caused a maximum 10% relaxation of phenylephrine-induced tone. A concentration–response curve for the most active compound was subsequently constructed. In another series of experiments, rings were pretreated with the soluble guanylate cyclase inhibitor ODQ (1 μM) for 30 min before the addition of phenylephrine. Pre-incubation with ODQ caused a 20% increase in phenylephrine response and a concentration–response curve for the most active compound was constructed. Relaxations were then evaluated as a percentage of the response to phenylephrine in the presence of either IBMX or ODQ, taken as 100%.

Effect on phorbol 12-myristate-13-acetate-induced contraction

Protein kinase C plays a key role in developing and/or maintaining vascular smooth muscle tone. Therefore, rings were stimulated with 1 μM phorbol 12-myristate-13-acetate (a protein kinase C stimulator) in the presence of extracellular Ca^{2+}. When the contractile response to phorbol 12-myristate-13-acetate had reached its maximum, the most active compound was added cumulatively (1–100 μM). Responses were then evaluated as a percentage of the initial response to phorbol 12-myristate-13-acetate, taken as 100%.

Solutions and chemicals

PSS containing (in mM) 118 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4 \cdot 7H_2O$, 1.2 KH_2PO_4, 25 $NaHCO_3$ and 11.5 glucose was bubbled with a 95% O_2/5% CO_2 gas mixture to create a pH of 7.4. PSS containing KCl at a concentration greater than 4.8 mM was prepared by replacing NaCl with equimolar KCl.

The compounds assayed in this work were isolated from several representatives of two of the three regional subfamilies of the Hyacinthaceae. (*R*)-5,6-Dihydroxy-7-methoxy-3-(4′-hydroxybenzyl)chroman-4-one (compound **1**), (*R*)-3′,5-dihydroxy-4′,7-dimethoxyspiro [2H-1-benzopyran-3(4H),7′-bicyclo[4.2.0]-octa[1,3,5]-trien]-4-one (compound **3**) and (*R*)-3′,5,7-trihydroxy-4′-methoxyspiro[2H-1-benzopyran-3 (4H),7′-bicyclo[4.2.0]-octa[1,3,5]-trien]-4-one (compound **4**), were isolated from *Drimiopsis maculata* (Koorbanally et al 2001, 2006b). (*R*)-Scillascillin (compound **2**) was isolated from *Eucomis schijffii* (Koorbanally et al 2006a). 6α-Hydroxy-4(14)-eudesmen-1-one (compound **5**) (Koorbanally et al 2005) and 14β-hydroxybufa-3,5,20,22-tetraenolide (compound **6**) were isolated from *Urginea epigea* (Koorbanally et al 2004), whilst urginin (compound **7**) was isolated from *Drimia altissima* (Pohl et al 2000). The compounds were shown to be pure by GC-MS analysis. Isoprenaline, IBMX, tetraethylammonium chloride, phenylephrine, phorbol 12-myristate-13-acetate, and nifedipine were from Sigma Chimica (Milan, Italy); ryanodine and ODQ from Calbiochem (La Jolla, CA); sodium nitroprusside from Riedel-De Haën AG (Seelze-Hannover, Germany). Compounds **1–7**, IBMX, phorbol 12-myristate-13-acetate, ODQ, and ryanodine, dissolved directly in dimethyl sulfoxide, and nifedipine, dissolved in ethanol, were diluted at least 1000-times before use. The resulting concentrations of dimethyl sulfoxide and ethanol (below 0.1%, v/v) failed to alter the response of the preparations. Tetraethylammonium chloride, isoprenaline and sodium nitroprusside were dissolved in distilled water.

Statistical analysis

Acquisition and analysis of data were accomplished by using GraphPad Prism version 4.03 (GraphPad Software, USA). Data are reported as mean ± s.e.m.; n was the number of rings processed (indicated in parentheses), isolated from at least three animals. Statistical analyses and significance as measured by either analysis of variance (followed by Dunnett's post tests) or Student's *t*-test for either paired or unpaired samples (two-tail) were obtained using GraphPad InStat version 3.06 (GraphPad Software, USA). In all comparisons, $P < 0.05$ was considered significant.

The pharmacological response to each substance was given as pIC50 (M).

Results

Effects of compounds 1–7 on rings precontracted with K60 or phenylephrine

As shown in Figures 2 and 3, compound **1** had no effect on endothelium-denuded rings stimulated with either K60 or phenylephrine. Compounds **5, 6**, and **7** caused a modest

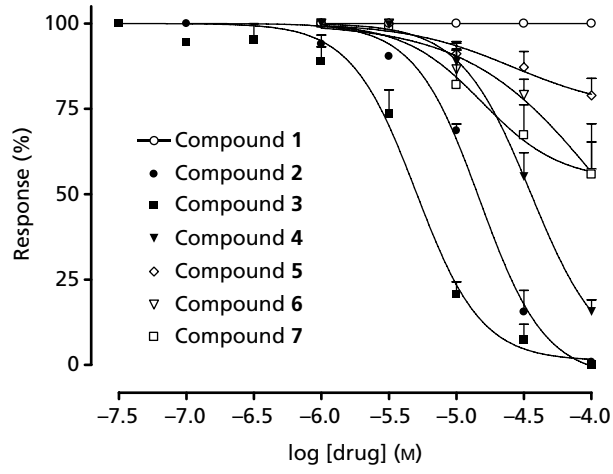

Figure 2 Effect of compounds **1–7** on endothelium-denuded rings depolarized with K60. In the ordinate scale, relaxation is reported as a percentage of the initial tension induced by K60, taken as 100%. Data points are mean ± s.e.m. (n = 3–5).

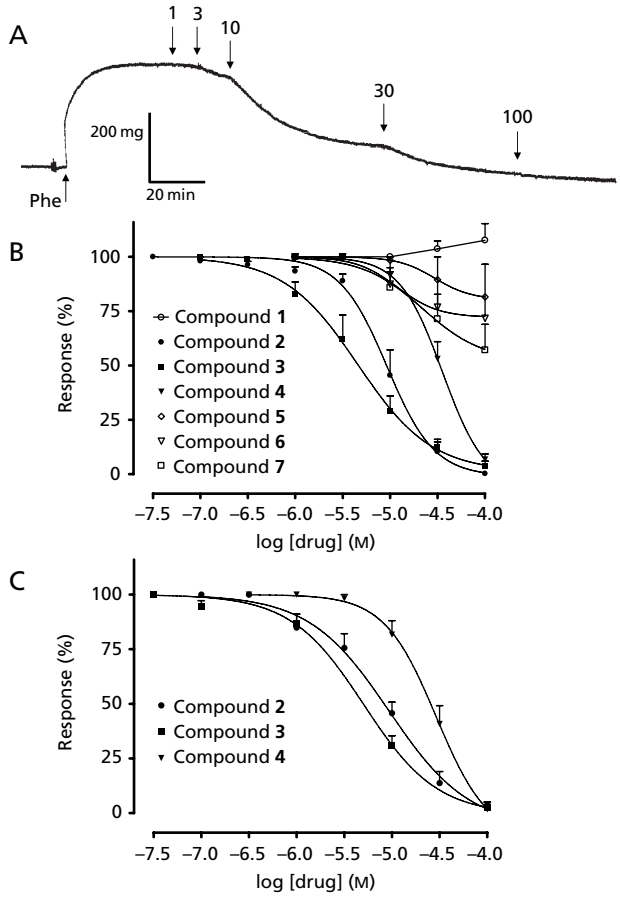

Figure 3 Effect of compounds **1–7** on rings precontracted with 0.3 μM phenylephrine. (A) Trace (representative of five similar experiments) of relaxation developed in response to cumulative concentrations (μM) of compound **3** added at the plateau of phenylephrine (Phe)-elicited contraction (endothelium-denuded ring). Concentration–response curves for compounds **1–7**, either in (B) endothelium-denuded or (C) for compounds **2, 3** and **4**, endothelium-intact rings. In the ordinate scale, relaxation is reported as a percentage of the initial tension induced by phenylephrine, taken as 100%. Data points are mean ± s.e.m. (n = 3–7, both endothelium-denuded and -intact).

concentration-dependent relaxation, which, however, never exceeded 50% of the tone attained under control conditions. Compounds **2, 3** and **4**, however, reverted, in a concentration-dependent manner, the contraction induced by both K60 and phenylephrine. pIC50 values of compounds **2, 3** and **4** did not differ significantly among the three model systems (endothelium-denuded rings stimulated with either K60 or phenylephrine, and endothelium-intact rings stimulated with phenylephrine), compound **3** proving to be the most active (Table 1).

Assessment of K^+-channel opening activity of compound 3

Under different conditions of high K^+-evoked depolarization (K25 or K60, respectively), the antispasmodic activity of compound **3** was comparable (Figure 4). Pre-incubation of the rings with 10 mM tetraethylammonium, while increasing

Table 1 Inhibition by homoisoflavonoids of the contraction elicited in aorta rings by high K^+ or phenylephrine

	K^+	Phenylephrine	
	−endothelium pIC50 (M)	−endothelium pIC50 (M)	+endothelium pIC50 (M)
Compound **2**	4.84 ± 0.03 (4)	5.08 ± 0.13 (6)	5.13 ± 0.09 (7)
Compound **3**	5.31 ± 0.05 (5)	5.40 ± 0.16 (6)	5.28 ± 0.08 (6)
Compound **4**	4.45 ± 0.05 (4)	4.47 ± 0.05 (6)	4.60 ± 0.03 (3)

Data represent mean ± s.e.m. Number of experiments is given in parentheses.

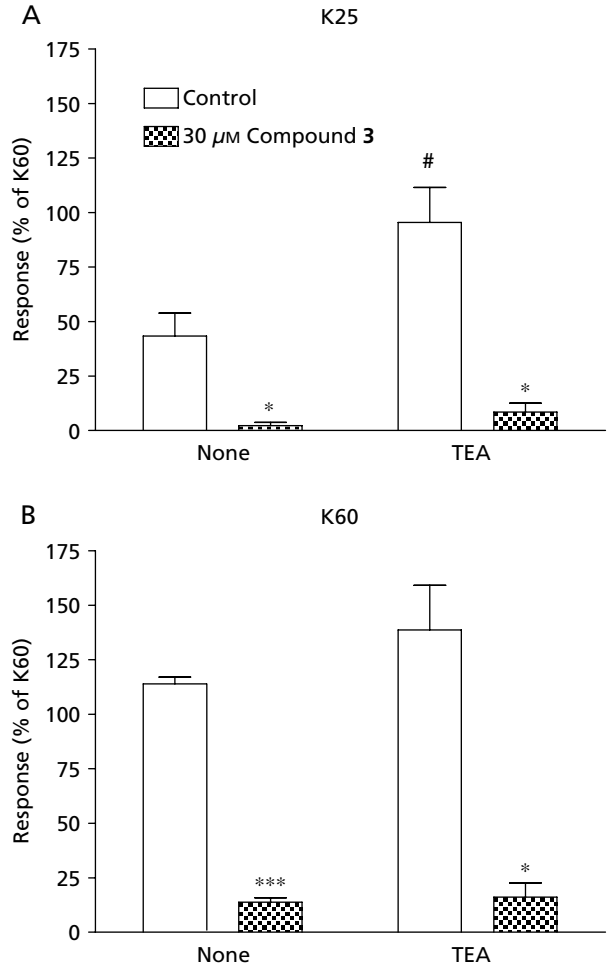

Figure 4 Effect of compound **3** on endothelium-denuded rings depolarized with either 25 (K25) or 60 mM extracellular K^+ (K60). Ca^{2+}-induced contractions were obtained in PSS containing (A) 25 (K25) or (B) 60 mM K^+ (K60), either in the absence (none) or in the presence of 10 mM tetraethylammonium (TEA). Compound **3** was added 30 min before the second challenge with either K25 or K60. Responses represent the percentage of the maximal contraction elicited by K60 when testing rings' contractile activity, taken as 100%. Columns represent mean ± s.e.m. (n = 4–5). *$P < 0.05$, ***$P < 0.001$ vs control (Student's *t*-test for paired samples); #$P < 0.05$ vs none control (Student's *t*-test for unpaired samples).

significantly only the response to K25, did not modify the antispasmodic effect of compound **3**.

Effects of compound 3, ryanodine, and nifedipine on Ca^{2+} release from the intracellular stores and on extracellular Ca^{2+} influx induced by phenylephrine

As shown in Figure 5, when rings had been pretreated with compound **3**, a concentration-dependent inhibition of contraction elicited by phenylephrine in Ca^{2+}-free medium was observed (see inset; estimated pIC50 = 4.66). Under the same experimental conditions, 30 μM ryanodine, but not 300 nM nifedipine, was able to antagonize the phenylephrine-induced contraction. When external Ca^{2+} was restored, with phenylephrine still present, both compound **3** (pIC50 = 5.14) and nifedipine significantly inhibited the subsequent contraction, whereas ryanodine stimulated it.

Effect of compound 3 on relaxation induced by isoprenaline or sodium nitroprusside in aortic rings precontracted with phenylephrine

Tension of rings elicited by 0.3 μM phenylephrine (272.8 ± 26.6 mg, n = 14) was not affected by pre-incubation with either 3 μM (243.0 ± 25.6 mg, n = 13) or 10 μM compound **3** (321.6 ± 32.9 mg, n = 12; $P < 0.05$, Dunnett's post test). Furthermore, compound **3** was not able to contract rings during the pre-incubation period (data not shown).

Pre-incubation with compound **3** did not affect the maximal vasorelaxing response evoked by either isoprenaline (a β-adrenoceptor agonist) or sodium nitroprusside (a NO donor) on rings precontracted with 0.3 μM phenylephrine (data not shown). In contrast, compound **3** altered pIC50 values of both compounds, causing a significant leftward shift of the curves (isoprenaline: 7.00 ± 0.24, n = 8, control; 7.34 ± 0.25, n = 7, 3 μM compound **3**; 7.89 ± 0.27, n = 7, 10 μM compound **3**, $P < 0.05$, Dunnett's post test; sodium nitroprusside:

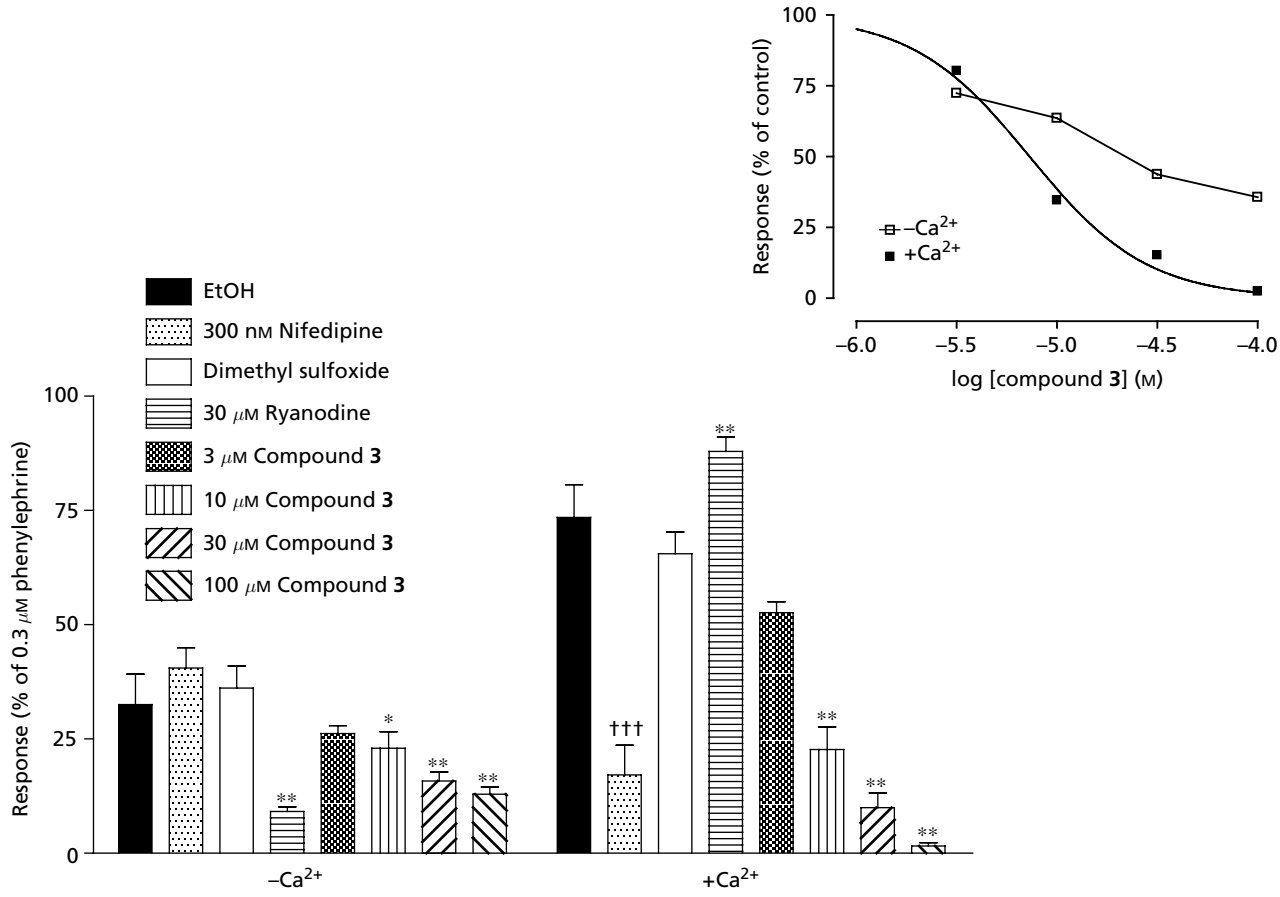

Figure 5 Effects of compound **3**, ryanodine, and nifedipine on Ca^{2+} release from the intracellular stores and on extracellular Ca^{2+} influx induced by phenylephrine in endothelium-denuded rings. Columns represent phenylephrine-induced contractions either in the absence ($-Ca^{2+}$) or in the presence ($+Ca^{2+}$) of extracellular Ca^{2+}, recorded under control conditions (vehicle only, ethanol or dimethyl sulfoxide, respectively) or in the presence of drug. These contractions were measured independently, the response to phenylephrine in the absence of extracellular Ca^{2+} representing the baseline for that obtained after the addition of extracellular Ca^{2+}. Columns are mean ± s.e.m. (n = 4–7) and represent the percentage of the response to 0.3 μM phenylephrine in PSS, taken as 100%. $^{\dagger\dagger\dagger}P < 0.001$ vs ethanol, Student's *t*-test for unpaired samples; $^{*}P < 0.05$, $^{**}P < 0.01$ vs dimethyl sulfoxide, Dunnett's post test. Inset: data for compound **3** were normalized to the corresponding control and plotted as a function of drug concentration.

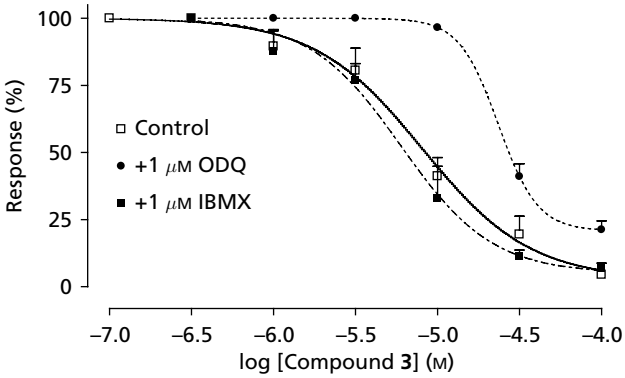

Figure 6 Relaxation induced by compound **3** in endothelium-denuded rings precontracted with phenylephrine in the absence or presence of ODQ or IBMX. While ODQ (1 μM) was pre-incubated for 30 min and left in contact with the preparation throughout the experiment, IBMX was added at the plateau of phenylephrine-induced contraction. In the ordinate scale, relaxation is reported as a percentage of the initial tension induced by phenylephrine, taken as 100%. Data points are mean ± s.e.m. (n = 5–8).

8.55 ± 0.12, n = 6, control; 8.70 ± 0.08, n = 6, 3 μM compound **3**; 9.10 ± 0.16, n = 5, 10 μM compound **3**, $P < 0.05$).

Effect of IBMX or ODQ on the relaxation induced by compound 3 in rings precontracted with phenylephrine

As shown in Figure 6, compound **3** inhibited, in a concentration-dependent manner, the contraction induced by phenylephrine (pIC50 = 5.13 ± 0.13, n = 8). Addition of the non-specific phosphodiesterase inhibitor IBMX did not affect the vasorelaxing effect of compound **3** (pIC50 = 5.22 ± 0.10, n = 7). On the contrary, the soluble guanylyl cyclase inhibitor ODQ (1 μM (Garthwaite et al 1995)) caused a rightward shift of the concentration–response curve of compound **3** (pIC50 = 4.62 ± 0.03, n = 6; $P < 0.01$, Dunnett's post test).

Effect of compound 3 on phorbol 12-myristate-13-acetate-induced contraction

Phorbol 12-myristate-13-acetate produced a slow but sustained contraction of rings incubated in normal PSS (291.6 ± 77.2 mg, n = 4). Compound **3** (1–100 μM), when added in the bath at the plateau of phorbol 12-myristate-13-acetate-induced contraction, produced a concentration-dependent relaxation (data not shown). The maximal effect of compound **3** recorded at 100 μM concentration was the reversion of approximately 50% of the contraction induced by the phorbol ester.

Discussion

We have demonstrated that, among the isolates from the Hyacinthaceae, only scillascillin-type homoisoflavonoids exhibited significant vasodilating effects on rat aortic rings. In particular, compound **3**, the most active of the series, displayed both antispasmodic and spasmolytic activity via a negative modulation of plasmalemmal Ca^{2+} influx responsible for the contraction of the vascular musculature. In contrast, compounds **1, 5, 6** and **7** elicited very weak vasodilation.

Contraction of smooth muscle is initiated, and to a lesser extent maintained, by a rise in the concentration of free Ca^{2+} in the cytosol originating from two intimately linked sources, the extracellular space (Ca^{2+} entry) and intracellular stores (Ca^{2+} release), located mostly in the sarcoplasmic reticulum. The former event occurs through a variety of Ca^{2+} permeable ion channels in the cytomembrane. The best-characterized Ca^{2+} entry pathway utilizes voltage-operated Ca^{2+} channels (VOCCs). The effects of the homoisoflavonoids **2, 3**, and **4** were investigated under experimental conditions that allowed for the identification of blockers of such channels. High K^+-induced contraction of rings, in fact, is the result of an increased Ca^{2+} influx through VOCCs and is specifically inhibited by Ca^{2+}-antagonists. Compounds **2, 3**, and **4** antagonized this contraction at levels comparable with the well-known Ca^{2+}-channel blocking drug nifedipine, indicating a VOCCs blockade.

K^+-channel openers represent a class of well-known vasodilating agents. The opening of K^+ channels increases K^+ efflux causing membrane potential hyperpolarization, which triggers the indirect closure of VOCCs thus leading to vasodilation (Nelson & Quayle 1995). The antispasmodic activity of K^+-channel openers, such as cromakalim (Norman et al 1994), is reduced when extracellular K^+ concentration is raised. Invariably, when the K^+ gradient across the membrane drops the effect of K^+-channel openers disappears (Gurney 1994). In contrast with cromakalim-induced antispasmodic effects (see Saponara et al 2004), however, compound **3**-induced activity was comparable under both low and high external K^+ concentrations. Furthermore, tetraethylammonium, a non-selective blocker of K^+ channels, failed to modify the antispasmodic activity of compound **3**. These data suggested that K^+ channels were not involved in the vasorelaxing action of compound **3**, although further experiments are needed to rule out fully their non-participation.

Compounds **2, 3** and **4** relaxed both intact and denuded rings precontracted with phenylephrine. Therefore, the participation of endothelium-derived vasodilators (e.g. NO) in their effect was excluded and not further investigated.

Smooth muscle contraction activated by α_1-adrenergic agonists derives from a combination of Ca^{2+} release from intracellular stores and Ca^{2+} entry from the extracellular space. The latter occurs through receptor-operated Ca^{2+} channels (ROCCs), activated by G-protein, and store-operated Ca^{2+} channels (SOCCs) activated by depletion of the Ca^{2+} stores (i.e. the sarcoplasmic reticulum). McFadzean & Gibson (2002) indicated that ROCCs and SOCCs may be closely related, being formed by proteins of the transient receptor potential channel family. The opening of ROCCs/SOCCs, beyond Ca^{2+} entry, results also in membrane depolarization and subsequent opening of VOCCs (Inoue et al 2001; Sanders 2001; Clapham et al 2003). Accordingly, the antagonism exerted by compounds **2, 3** and **4** on phenylephrine-induced contraction might result from the action on one of these targets or, more likely, from that on two or more of these targets.

The latter hypothesis was supported by the finding that these compounds relaxed preparations precontracted with either K60 or phenylephrine with similar efficacy and potency. In particular, compound **3** antagonized both release of Ca^{2+} from sarcoplasmic reticulum and phenylephrine-triggered Ca^{2+} influx, the latter with higher potency. This difference pointed once more to the Ca^{2+}-channel blocking activity of compound **3**. On the other hand, the reduced phenylephrine-induced contraction in Ca^{2+}-free medium by compound **3** suggested that it possibly depleted phenylephrine-sensitive Ca^{2+} stores during the pre-incubation period, or conversely antagonized inositol triphosphate-dependent Ca^{2+} release from intracellular stores. Contrary to ryanodine (Low et al 1993), in fact, compound **3** failed to elicit contraction in rings bathed in PSS (data not shown).

When extracellular Ca^{2+} was restored, still in the presence of phenylephrine, Ca^{2+} influx occurred through ROCCs/SOCCs, and VOCCs (see above), thus causing an additional tonic response. Compound **3** antagonized also Ca^{2+} influx in a concentration-dependent manner, thus suggesting that ROCCs/SOCCs, besides VOCCs, probably represented additional targets responsible for compound **3** antispasmodic activity. This was supported by the high structural homology between the pore-forming α_1-subunits of VOCCs and the proteins that form their receptor- and store-operated counterparts (McFadzean & Gibson 2002). In contrast with compound **3** and nifedipine, ryanodine seemed to increase Ca^{2+} entry, possibly by stimulating indirectly SOCCs.

Vasodilators, able to raise the cytoplasmic levels of cGMP or cAMP, are known to increase the potency of isoprenaline and/or sodium nitroprusside which trigger these pathways, so evoking smooth muscle relaxation. Compound **3** was shown to shift leftward both isoprenaline and sodium nitroprusside concentration-relaxation curves, giving support to the hypothesis of vasodilation mediated by the increase of intracellular concentrations of cyclic nucleotides. Moreover, the vasorelaxing effect of compound **3** was antagonized by pretreatment of rings with the selective inhibitor of soluble guanylyl cyclase ODQ. Therefore, the leftward shift of the concentration–relaxation curve for isoprenaline might have been due to a cross activation between the two second messenger systems (i.e. cGMP enhanced cAMP-mediated relaxation, possibly through the inhibition of the cGMP-inhibited phosphodiesterase III (Komas et al 1991)), which has been well documented in vascular tissue (Maurice et al 2003). In fact, in rat aorta, low concentrations of nitrovasodilators enhanced the relaxant effect of isoprenaline (Maurice et al 1991). Finally, threshold concentrations of IBMX, a non-selective phosphodiesterase inhibitor, failed to augment vascular smooth muscle relaxation induced by compound **3**. This was in agreement with the observation that IBMX improved the relaxation induced by cAMP- but not cGMP-forming drugs (Fujimoto & Matsuda 1990).

cGMP-induced smooth muscle relaxation is mediated mainly by protein kinase G activation. It involves several molecular events that culminate in the reduction of intracellular Ca^{2+} concentration and a decrease in the sensitivity of contractile proteins to Ca^{2+} (Carvajal et al 2000). cGMP promotes a reduction of intracellular Ca^{2+} concentration through the activation of protein kinase G (PKG). Activated PKG phosphorylates several key target proteins, including ion channels (e.g. VOCCs), ion pumps (e.g. plasmalemma and sarcoplasmic reticulum Ca^{2+}-ATPases), receptors (e.g. IP_3 receptor), and enzymes (e.g. phospholipase C), all involved in the control of intracellular Ca^{2+} concentration. Phosphorylation of these target proteins reduced intracellular Ca^{2+} concentration and resulted in relaxation of smooth muscle. On the other hand, cGMP reduced the sensitivity of the contractile system to Ca^{2+} by stimulating myosin light chain phosphatase as well as by inhibiting protein kinase C activity. The latter was consistent with the relaxation produced by compound **3** of phorbol 12-myristate-13-acetate-induced tone, which depended on an increased sensitivity of the contractile apparatus to Ca^{2+} (Jiang & Morgan 1987). Taken together, all these actions partly explain the effects of compound **3** on rat aortic rings.

Conclusions

Homoisoflavonoids with a 3-spiro-cyclobutene ring may represent novel vasodilators, or at least provide scaffolds for the design of novel vasoactive agents. Accordingly, the vasodilating effect of homoisoflavanoids might validate their inclusion in traditional preparations to treat ailments associated with vasoconstriction.

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