Vasodilatation Produced by Orientin and Its Mechanism Study

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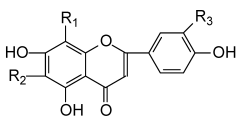
In this paper we investigated the vascular activity and possible mechanism of Orientin, from bamboo leaves (*Phyllostachys nigra*), in isolated thoracic aortic rings from New Zealand rabbit. Among the four compounds, studied, only Orientin relaxed phenylephrine-induced contractions with an IC_{50} value of 2.28 μM in the endothelium intact and with an IC_{50} value around 7.27 μM in the endothelium removed aortic rings. The vasorelaxant effect of Orientin on endothelium-intact thoracic aortic rings was attenuated by the nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine methyl ester, but not by indomethacin (a cyclooxygenase inhibitor), tetraethylammonium chloride (K^+ channels inhibitor) or propranolol (β-receptor inhibitor). Furthermore, Orientin inhibited norepinephrine (NE), $CaCl_2$ and KCl-induced vasoconstriction concentration dependently in a non-competitive manner, and also reduced both the initial fast release and the sustained phases of phenylephrine-induced contractions. Orientin can stimulate NO production from endothelial cells. Orientin also increased cyclic guanosine 3,5-cyclic monophosphate (cGMP) levels without changes in adenosine-3′,5′-cyclic phosphoric acid (cAMP) in rabbit aorta. The results showed that Orientin relaxed thoracic aortic rings by the nitric oxide-cGMP pathway, and in the vascular smooth muscle inhibited the contraction induced by the activation of receptor-operating and voltage-dependent Ca^{2+} channels. Cyclooxygenase pathway, potassium channels, β-receptors and cAMP pathway, on the other hand, had no apparent roles. The inhibition of both intracellular Ca^{2+} release and extracellular Ca^{2+} influx may be one of the main vasorelaxant mechanisms of Orientin.

Key words orientin; vasorelaxant; endothelium; nitric oxide (NO); rabbit thoracic aorta; cyclic nucleotide

Bamboo leaves, *Phyllostachys nigra* (Lodd. ex. lind-1 Munro. L), have been used as a Chinese medicament for thousands of years. Theses leaves have anti-free radical activity and are comparable with the leaf of *Ginkgo bilabo*, which is one potential resource for natural antioxidant and free radical scavenger.[1] Our previous investigation revealed that bamboo leaves have significant effects on protecting myocardial ischemia in rat, restraining blood platelet aggregation of rabbit, increasing coronal flow and have a vasorelaxant effect on rabbit thoracic aortic rings, and also reduce arterial blood pressure. Here, we further investigated the vasorelaxant effect of four flavonoid C-glycosyl compounds (Fig. 1): Orientin (Luteolin-8-C-glucoside), Isoorientin (Luteolin-6-C-glucoside), Isovitexin (Apigenin-6-C-glucoside), and Vitexin (Apigenin-8-C-glucoside), first isolated from bamboo leaves, all of which are also found in numerous plants.[2,3] It is reported that these flavonoids possess anxiolytic properties[4,5] and that Orientin possesses antioxidative activity,[6] however, there is no information about the vasorelaxant effect of these compounds. In the present investigation, we examined the vasodilatation effects of these flavonoid C-glycosyl compounds and found only Orientin produced a vasorelaxant effect on rabbit aortic rings. The data from the present study suggested that Orientin relaxed thoracic aortic rings by the nitric oxide-guanosine 3,5-cyclic monophosphate (cGMP) pathway, and in the vascular smooth muscle inhibited the contraction induced by the activation of receptor-operating and voltage-dependent Ca^{2+} channels. Cyclooxygenase pathway, potassium channels, β-receptors and adenosine-3′,5′-cyclic phosphoric acid (cAMP) pathway, on the other hand, had no apparent roles. The inhibition of both intracellular Ca^{2+} release and extracellular Ca^{2+} influx may be one of the main vasorelaxant mechanisms of Orientin.



Name	R1	R2	R3
Orientin	Glu	H	OH
Isooorientin	H	Glu	OH
Vitexin	Glu	H	H
Isovitexin	H	Glu	H

Fig. 1. Chemical Structure of Flavonoid C-Glycosyl

MATERIALS AND METHODS

Animal New Zealand rabbits were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University.

Drugs Orientin, Isoorientin, Vitexin, Isovitexin were purchased from Extrasynthese (Genay France), norepinephrine, N^G-nitro-L-arginine methyl ester (NAME), indomethacin and tetraethylammonium chloride were purchased from Sigma (St. Louis, MO, U.S.A.), and propranolol from No. 2 Pharmacy Factory (Beijing, China). Acetylcholine chloride was purchased from the Medicinal Materials Service Station of the Military Medicine Academy of Sciences (China). Phenylephrine hydrochloride was from Huida

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Pharmaceutical Factory of Datong (China), and nitric oxide (NO) assay kit was from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). cAMP and cGMP radioimmunoassay kits were from the Isotope Department of Shanghai University of Traditional Chinese Medicine (Shanghai, China).

Preparation of the Isolated Aorta New Zealand rabbits (2.0—2.5 kg) were sacrificed by decapitation. The thoracic aorta was excised and adhesive connective tissues were carefully cleared away. The aorta was cut into approximately 3—4 mm-long ring segments. Denuded aorta ring segments were prepared by mechanical method with cotton pipe-cleaners. The denudation was confirmed by the absence of relaxation in response to direct application of acetylcholine (Ach) 3 μM. The isolated aorta ring was mounted in a 10 ml organ bath containing Krebs' solution continuously aerated with a gas mixture of 95% O_2 and 5% CO_2 and maintained at 37 °C. The composition of Krebs' solution was as follows: 118 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.5 mM $MgSO_4$, 2.5 mM $CaCl_2$ and 11.1 mM glucose. Isometric tension change was measured with a force-displacement transducer and recorded by a bio-signal recording system (Ms-2000, Guangdong College of Pharmacy, Guangzhou, China). Before starting the experiment, all preparations were allowed to equilibrate for 60 min, during which time Krebs' solution was replaced twice.

Establishment of Concentration–Contractile Response Curves of the Four Compounds To evaluate the vasorelaxant and endothelium-dependent effects of Orientin, Isoorientin, Vitexin, and Isovitexin, endothelium-intact and -denuded preparations were pretreated with phenylephrine (PE, 10 μM) to produce sustained contractions.[7,8] Lack of functional vascular endothelium was confirmed by the loss of relaxant response to 3 μM acetylcholine before the experiment began.[9] After the contraction had reached a stable plateau, cumulative concentrations of test compound were added. The vasorelaxant effect was expressed as a percentage of relaxation and the IC_{50} (the concentration to produce a 50% maximal relaxation) value was determined from the concentration–response curve by data fitting with computer software GraFit (Erithacus Software, Staines, Middlesex, U.K.).

Examination of Mediator for Endothelium-Related Vasorelaxation The involvement of the mediator for endothelium-related vasorelaxation induced by test compound was examined by pretreatment of preparations with N^G-nitro-L-arginine methyl ester (a NO synthase inhibitor), tetraethylammonium chloride (K^+ channels inhibitor), propranolol (β-receptor inhibitor) or indomethacin (a cyclooxygenase inhibitor).[7]

Measurement of NO ECV304, a human umbilical vein endothelium cell line, was obtained from the cell bank of the Chinese Academy of Sciences and was grown in 35-mm^2 dishes. Upon reaching confluence, the medium was changed to Hanks' balanced salt solution (HBSS) with L-arginine (100 μM) and $CaCl_2$ (to 2.5 mM) was added. The change to HBSS was necessary because it provided the least interference in the assay. However, additional Ca^{2+} was required to make the final concentration comparable to that in normal Krebs' solution.[10] The cells were then equilibrated for 60 min at 37 °C. The total content of NO in the medium before drug treatment was calculated and taken as 100%. Vehicle or Orientin (3, 10, 30 μM) was then added for 10 min to stimulate NO release. The amount of NO released by cells was determined using a NO assay kit according to the manufacturer's protocol.[11] To study whether Orientin-induced NO release was dependent on extracellular Ca^{2+}, similar experiments also were carried out in Ca^{2+}-free HBSS containing EGTA (2 mM). The absorbance of sample was read at 550 nm.

Cyclic Nucleotide Levels The method was essentially the same as reported previously.[12,13] Aortic rings were isolated as described above. The tissues were incubated in Krebs' solution with Orientin (3, 10, 30 μM) or vehicle at 37 °C for 10 min. The cyclic nucleotide phosphodiesterase inhibitor 3-isobutyl-1-methylaxanthine (10 μM) was added during the last 5 min of the incubation period. The reaction was stopped by immersing the tissue in liquid nitrogen and storing at −80 °C up to the time of thawing in chilled 6% trichloroacetic acid. The thawed tissues were homogenized and centrifuged at 10000 ***g*** for 5 min. The supernatant fractions were extracted four times with 5 volumes of water-saturated diethyl ether, dried under a stream of nitrogen, and assayed for cGMP and cAMP content by radioimmunoassay, (radioimmunoassay kits from the Isotope Department of Shanghai University), and the precipitate was saved for protein determination.[14] The cGMP and cAMP levels were expressed as pmol/mg protein.

Assessment of Cumulative Concentration–Response Curves with Norepinephrine (NE), $CaCl_2$ and KCl A series of experiments was designed to assess the involvement of α-adrenoceptors in the vasorelaxant effect of test compound in endothelium-denuded aortic preparations.[15,16] Various concentrations of Orientin (3, 10, 30 μM) were added 10 min before the construction of cumulative concentration-response curves with NE (3 nM to 10 μM), $CaCl_2$ (3 μM to 3 mM) and KCl (1—64 mM). The results were expressed as the percentage of the maximum contractile tension to NE before and after pretreatment with Orientin.

Detection of Initial Fast and Sustained Phases Induced by Phenylephrine The contractile response of endothelium-denuded aortic ring to phenylephrine can be separated into initial and sustained phases according to the method described by Chiou *et al*.[17] The initial contraction was first initiated with phenylephrine (30 μM) in Ca^{2+}-free Krebs' solution (containing 2.5 mM EGTA) and the sustained contraction was then induced by further addition of 2.5 mM $CaCl_2$. Orientin was pretreated 10 min prior to phenylephrine addition.

Statistical Analysis Data are expressed as the means ± S.D. and were analyzed by Student's *t*-test with a significance level of $p<0.05$. IC_{50} values were calculated by the computer software GraFit.

RESULTS

Concentration–Contractile Response Curves of the Four Compounds Orientin (0.1—30 μM) concentration-dependently relaxed endothelium intact rings precontracted with phenylephrine (PE, 10 μM), while Isoorientin (0.1—30 μM), Vitexin (0.1—30 μM), and Isovitexin (0.1—30 μM) had no effect on endothelium-intact or -denuded rings precontracted with phenylephrine (PE, 10 μM). The vasorelaxant responses to Orientin were significantly depressed in endothe-

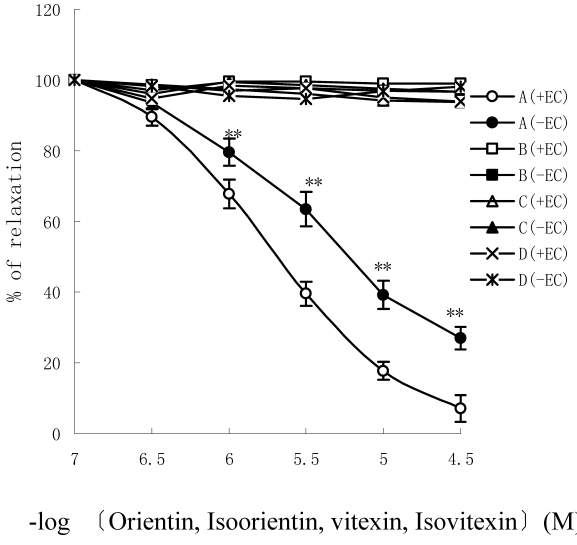

Fig. 2. A: Orientin (0.1—30 μM), B: Isoorientin (0.1—30 μM), C: Vitexin (0.1—30 μM), D: Isovitexin (0.1—30 μM) on phenylephrine induced contractions in isolated rabbit endothelium intact (+EC)- and denuded (−EC)-thoracic aortic rings. When phenylephrine (PE, 10 μM) induced contractions reached the plateau, the test compound was cumulatively added. Orientin relaxed phenylephrine-induced contractions with an IC_{50} value of 2.28 μM in the endothelium-intact and with an IC_{50} value around 7.27 μM in the endothelium-removed aortic rings. Data are means±S.D. (n=5). Data are expressed as percentage of the control tension. $^{**}p<0.01$, as compared with the +EC.

lium-denuded preparations (Fig. 2). The IC_{50} values of Orientin for PE-induced concentrations were significantly increased 3.19-fold in endothelium denuded preparations as compared with these in endothelium-intact preparations.

Cumulative Concentration–Response Curves with NE, $CaCl_2$ and KCl The maximum responses of the cumulative concentration–response curves to norepinephrine, $CaCl_2$, or KCl were concentration-dependently depressed by Orientin (3, 10, 30 μM). These results indicated a non-competitive antagonism (Fig. 3).

Mediator for Endothelium-Related Vasorelaxation Similar to endothelium removal, the vasorelaxant effects of Orientin on PE-induced contractions in endothelium-intact aortic preparations were attenuated by pretreatment with N^G-nitro-L-arginine methyl ester (500 μM, 10 min), but not by indomethacin (30 μM, 45 min), TEA (10 mM, 60 min) or by propranolol (10 μM, 15 min) (Fig. 4).

Effects of Orientin on NO Production Orientin (3, 10, 30 μM) concentration dependently stimulated NO production from endothelial cells in HBSS containing 2.5 mM Ca^{2+} but not in Ca^{2+}-free HBSS (Fig. 5).

Effects of Orientin on Cyclic Nucleotide Levels Treatment with Orientin (3, 10, 30 μM) showed a concentration-dependent increase in cGMP levels, whereas the same treatment had an insignificant effect on cAMP levels (Fig. 6).

Initial Fast and Sustained Phases Induced by Phenylephrine Both the initial constriction produced by phenylephrine in calcium-free medium with EGTA and the sustained constriction resulting from the reintroduction of $CaCl_2$ to the medium were all concentration dependently attenuated with Orientin (3, 10, 30 μM) pretreatment (Fig. 7).

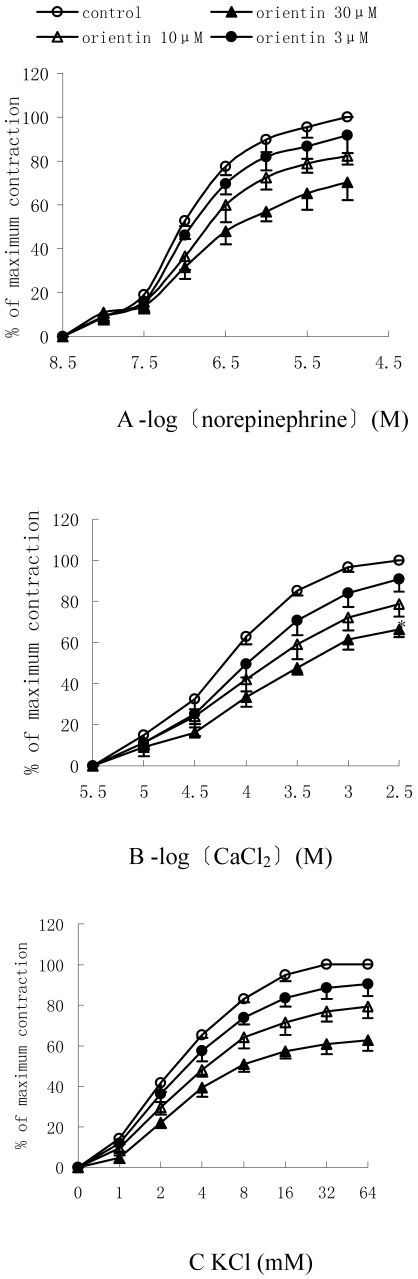

Fig. 3. Effect of Orientin on the Concentration–Response Curves of Norepinephrine, $CaCl_2$, and KCl in Endothelium-Denuded Aortic Preparations

Orientin (3, 10 or 30 μM) was added 10 min prior to construction of the concentration–response curve of norepinephrine (A), $CaCl_2$ (B), or KCl (C). Data are means± S.D. (n=5) and are expressed as % of maximum contraction.

DISCUSSION

Among the four compounds of bamboo leaves, only Orientin produced vasorelaxant effects on rabbit aortic rings. The data from the present study suggested that this vascular relaxant effect was attributable to its action on the endothelium and on the vascular smooth muscles.

Endothelium removal had great influence on the vasorelaxant effect of Orientin in PE-induced contractions. The most potent mediator known are the vasodilators, endothelium-derived relaxing factor (NO), prostacyclin (PGI_2), and β-receptor agonist. The use of different blockers (N^G-nitro-L-arginine methyl ester, indomethacin and propranolol) to ex-

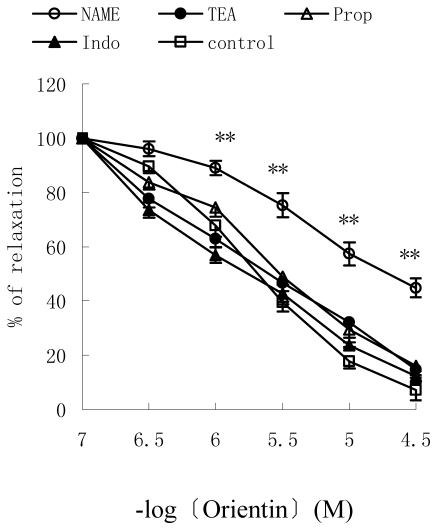

Fig. 4. Effect of N^G-Nitro-L-arginine Methyl Ester, Propranolol and Indomethacin on the Vasorelaxant Action of Orientin (0.1—30 μM) in Endothelium-Intact Aortic Preparations Precontracted with Phenylephrine (PE, 10 μM)

Preparations were pretreated with N^G-nitro-L-arginine methyl ester (500 μM, 10 min), indomethacin (30 μM, 45 min) or by Propranolol (10 μM, 15 min) prior to Orientin addition. Data are means±S.D. (n=5) and expressed as % of control tension. $^{**}p<0.01$, as compared with the control.

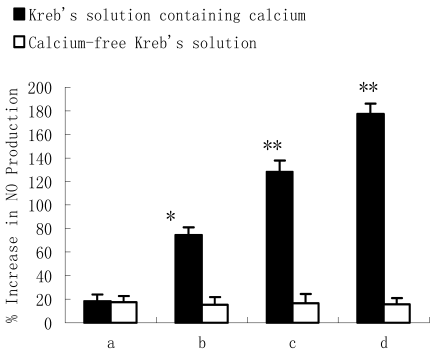

Fig. 5. Effects of Orientin (3, 10 or 30 μM) on NO Production

Orientin (3, 10 or 30 μM) significantly stimulated NO production in ECV304 in HBSS containing Ca^{2+} in a concentration-dependent manner. It did not significantly alter the NO content in Ca^{2+}-free plus EGTA (2 mM) HBSS. Data are means±S.D. (n=6). $^{*}p<0.05$, $^{**}p<0.01$, as compared with the control.

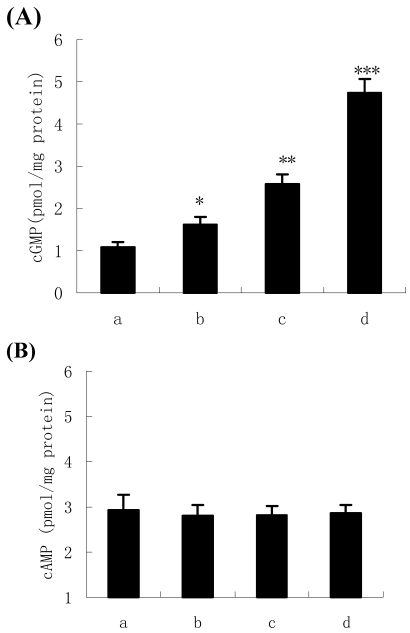

Fig. 6. Effects of Orientin on cGMP and cAMP Accumulation in Endothelium-Intact Rabbit Aorta

(A) cGMP levels. (B) cAMP levels. Treatment with Orientin (3, 10, 30 μM) showed a concentration-dependent increase in cGMP levels, whereas the same treatment had an insignificant effect on cAMP levels. Data are means±S.D. (n=10). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, as compared with the control.

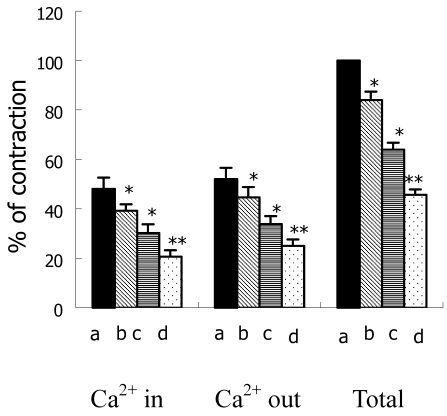

Fig. 7. Effects of Orientin (3, 10 or 30 μM) on the Two Compontents of the Contraction Induced by PE (30 μM)

Data are means±S.D. (n=6). $^{*}p<0.05$, $^{**}p<0.01$, as compared with the control.

amine the possible involvement in endothelium-related vasorelaxation, showed the relaxing action of Orientin was attenuated in endothelium-denuded and endothelium-intact aorta pretreated with L-NAME. This suggested that the vasorelaxant effect of Orientin was, at least in part, dependent upon the endothelium, and involvement of a mediator in the Orientin-induced endothelium-related vasorelaxation indicated that NO was involved. The action of Orientin on endothelial cells to release NO was confirmed by an experiment directly on the endothelial cells. Although the underlying mechanism of action was unclear, external Ca^{2+} was necessary for the action of Orientin to increase NO release from endothelial cells. NO is a potent vasodilator produced by the endothelium under basal conditions and in response to a variety of agonists. It diffuses from the endothelium to the underlying vascular smooth muscle, where it causes relaxation through the activation of soluble guanylate cyclase, causing an increase in 3,5-cyclic guanosine monophosphate.[18] Orientin increases cGMP levels without changes in cAMP in rabbit aorta. These results suggest that the relaxation of the rabbit aorta caused by Orientin may be mediated, at least in part, through the activation of the NO-cGMP-dependent signaling pathway. There are at least three widely distributed intracellular proteins known to bind cGMP,[19] namely, the cGMP-dependent protein kinase, the cGMP-stimulated cAMP phosphodiesterase, and the cGMP-binding protein-phosphodiesterase. Furthermore, specific tissues are known to contain novel cGMP binding protein, such as the cGMP-dependent cation channel protein.[20] Because elevations in intracellular cGMP could evoke changes in the activity of any

of these proteins, further investigation is needed to clarify this mechanism in detail. The vasorelaxation caused by Orientin in intact aorta was shown to persist in the presence of indomethacin (which blocks the formation of PGI_2 by inhibiting cyclooxygenase), propranolol (β-receptor inhibitor), implying that this effect was not mediated by prostacyclin or β-receptor. Tetraethylammonium chloride (TEA) is a Ca^{2+}-sensitive K^+ channels inhibitor, and the vasorelaxation caused by Orientin in intact aorta was shown to persist in the presence of TEA, implying that this effect was not mediated by K^+ channels. Taken together, these results suggest that the relaxation of the rabbit aorta caused by Orientin may be mediated through the activation of the NOS-guanylate cyclase pathway.

Indeed, pretreatment with Orientin (0.1—30 μM) inhibited norepinephrine (NE)-induced contractions in endothelium-denuded aortic rings, supporting a direct action on the vascular smooth muscle cells. Furthermore, Orientin inhibited NE, $CaCl_2$ and KCl-induced vasoconstriction concentration dependently in a non-competitive manner. The fact that it inhibited NE-induced vasoconstriction indicated that Orientin inhibited receptor-operating calcium channels, because NE can open receptor-operating calcium channels[21,22]; its inhibition of $CaCl_2$ and KCl-induced vasoconstriction concentration dependently and non-competitively, indicatd that it inhibited voltage-dependent Ca^{2+} channels, for high K^+ can excite voltage-dependent Ca^{2+} channels and increase Ca^{2+} influx.[23] Thus the results suggest that Orientin blocks both voltage-dependent Ca^{2+} channels and receptor-operating Ca^{2+} channels.

It has been suggested that there are biphasic responses, including fast and slow components in the vasoconstriction induced by the α_1 adrenoceptor agonist, phenylephrine.[24—26] The fast phase is due to the release of intracellular Ca^{2+}, and the sustained phase is largely dependent on the influx of external Ca^{2+}. The results showed that Orientin inhibited the fast contraction produced by phenylephrine in calcium-free medium and the sustained contraction resulting from the reintroduction of $CaCl_2$ to the medium. Therefore, Orientin had effects on both phenylephrine-sensitive intracellular Ca^{2+} release and phenylephrine-induced Ca^{2+} influx, and the inhibition of both phenylephrine-sensitive intracellular Ca^{2+} release and phenylephrine-induced Ca^{2+} influx may be one of the main vasorelaxant mechanisms of this compound.

In conclusion, the present findings suggest that Orientin relaxed thoracic aortic rings by the nitric oxide-cGMP pathway, and in the vascular smooth muscle inhibited the contraction induced by the activation of receptor-operating and voltage-dependent Ca^{2+} channels. Cyclooxygenase pathway, potassium channels, β-receptors and cAMP pathway, on the other hand, had no apparent roles. The inhibition of both intracellular Ca^{2+} release and extracellular Ca^{2+} influx may be one of the main vasorelaxant mechanisms of Orientin.

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