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Vasodilator effects of visnagin in isolated rat vascular smooth muscle

Juan Duarte [a], Francisco Pérez-Vizcaíno [b,*], Ana Isabel Torres [a], Antonio Zarzuelo [a], José Jiménez [a], Juan Tamargo [b]

[a] *Department of Pharmacology, School of Pharmacy, University of Granada, 18071 Granada, Spain*
[b] *Department of Pharmacology, School of Medicine, University Complutense of Madrid, 28040 Madrid, Spain*

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Abstract

Visnagin (4-methoxy-7-methyl-5*H*-furo [3,2-*g*][1]-benzopyran-5-one) is an active principle of the fruit of *Ammi visnaga*, a plant traditionally used in cardiovascular disorders. We have studied its vasodilator effects in rat vascular smooth muscle. The results demonstrated that visnagin inhibited the contractile responses induced in rat aortic rings by: (a) KCl or increases of extracellular Ca^{2+} in KCl depolarized aortic rings, its effects being more potent against low (20 mM) than high (80 mM) KCl-induced contractions, (b) noradrenaline in Ca^{2+}-containing solution and less effectively those in Ca^{2+}-free solution and (c) phorbol 12-myristate 13-acetate (PMA) in a Ca^{2+}-containing and with a lower potency in Ca^{2+}-free medium. The relaxation induced by visnagin in aorta precontracted with noradrenaline was not affected by endothelium removal. Additionally, visnagin inhibited the spontaneous myogenic contractions of portal veins. The results showed that visnagin inhibited vascular smooth muscle contractility by acting at multiple sites. In the range of 10^{-6} M to 5×10^{-5} M visnagin appears to inhibit only the contractions mediated by Ca^{2+} entry through pathways with low sensitivity to classical Ca^{2+}-entry blockers, i.e. agonist-, PMA- or mild depolarization-induced Ca^{2+} entry. Therefore, the vasodilator profile of visnagin, is not that of typical Ca^{2+}-entry blockers which preferentially inhibit the contractions induced by strong depolarizations. At higher concentrations ($> 5 \times 10^{-5}$ M) visnagin causes non-specific inhibition of vascular smooth muscle contractility.

Keywords: Visnagin; Aorta, rat; Portal vein

1. Introduction

The fruit of *Ammi visnaga* (L.) Lamarck (Apiaceae) exhibit peripheral and coronary vasodilator activity and relax various smooth muscles and, therefore, have been traditionally used for the treatment of angina pectoris and as antispasmodic agents (Anrep et al., 1946, 1949; Charlier, 1971; Weiss, 1985). The active principles extracted from these fruit included khellin, visnagin and visnadin (Charlier, 1971). In isolated rat aorta, khellin inhibited the contractions induced by noradrenaline and KCl as well as those induced by caffeine and noradrenaline in Ca^{2+}-free media (Ubeda and Villar, 1989; Ubeda et al., 1991). Moreover, it increased cAMP levels and reduced ^{45}Ca entry in aortic strips stimulated by KCl or noradrenaline without affecting the ^{45}Ca efflux stimulated by noradrenaline. From these results it was concluded that khellin, by acting at multiple sites of action, decreased the availability of Ca^{2+} required for smooth muscle contraction (Ubeda et al., 1991).

Visnagin (4-methoxy-7-methyl-5*H*-furo[3,2-*g*][1]-benzopyran-5-one) differs from khellin in that it lacks a methoxy group in position 2. Very recently, visnagin has been shown to relax KCl-and noradrenaline-induced contractions in guinea-pig aortic strips to a similar extent (Rauwald et al., 1994) and this vasorelaxant effect was explained by the inhibition of Ca^{2+} entry into vascular smooth muscle cells. However, the mechanism(s) responsible for the vasodilator action of visnagin is unknown. The present study was therefore undertaken to gain further information into the mechanisms implicated in its vasodilator effects in rat vascular smooth muscle.

* Corresponding author. Department of Pharmacology, School of Medicine, University Complutense of Madrid, 28040 Madrid, Spain. Tel. 34-1-3941472, fax 34-1-3941470.

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2. Materials and methods

2.1. Tissue preparation and tension measurement

Wistar rats (either sex, 250–300 g) were killed by a blow on the head. The descending thoracic aorta and portal veins were rapidly dissected and placed in a modified physiological saline solution (PSS) of the following composition (mM): NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2, KH_2PO_4 1.2 and glucose 11. After excess fat and connective tissue were removed, the aortae were cut into rings (4–5 mm in length) and portal veins into longitudinal segments of about 2 mm in width and 15 mm in length. Aortic rings were mounted under a tension of 1 g using two parallel L-shaped stainless-steel holders inserted into the lumen; portal vein segments were mounted vertically under a basal tension of 1 g in 10 ml organ baths containing PSS. Contractile responses were recorded isometrically by a force-displacement transducer (Letigraph 2000, Letica) connected to a Letica polygraph as previously described (Pérez-Vizcaíno et al., 1991; Duarte et al., 1994). The tissue bath was maintained at 37°C and bubbled with a 95% O_2-5% CO_2 gas mixture. For the experiments in which Ca^{2+}-free PSS was used, Ca^{2+} was omitted and 0.5 mM ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) was added. In some experiments the endothelium of the aorta was mechanically removed by gently rubbing the intimal surface. The absence of endothelium was confirmed by the absence of relaxing effects of acetylcholine (10^{-7} M–10^{-5} M) on aortic rings previously contracted with 10^{-7} M phenylephrine. Each preparation was allowed to equilibrate for at least 90 min prior to initiation of experimental procedures and during this period the incubation media were changed every 20 min before the addition of drugs.

After equilibration the following experiments were performed. (a) Aortic rings were exposed to single submaximal concentrations of KCl (20 or 80 mM) and noradrenaline (10^{-5} M) for 20 min. Control contractile responses to each agonist were obtained at the beginning of the experiment until two successive responses were almost identical in height. This was followed by exposure to visnagin for 15 min prior to the addition of KCl or noradrenaline and during re-exposure to the stimulatory agents. Only one agonist was used in each experiment. The results of these experiments are expressed as percentages of the maximal control agonist-induced contractile responses. (b) To determine whether visnagin could relax an existing contraction, aortic rings were contracted with single concentrations of noradrenaline (10^{-5} M), KCl (30 or 80 mM) or phorbol 12-myristate 13-acetate (PMA, 10^{-7} M). When the contractile response to each agonist was stable, visnagin was added in progressively increasing cumulative concentrations (10^{-6} M–10^{-4} M). Rings were allowed to reach a new steady-state tension before each successive addition of visnagin (aprox. 15 min). The ability of glibenclamide, a blocker of ATP-sensitive K^+ channels (Ashcroft, 1988), to antagonize the relaxant effect of visnagin was tested on the contractions induced by 30 mM KCl. Additionally, the relaxant effect of visnagin on noradrenaline-induced contractions was studied with aortae pretreated with tetraethylammonium. Glibenclamide and tetraethylammonium were added to the bath after the contractile responses induced by both stimulatory agents reached steady-state values and 20 min before the addition of visnagin. These results were expressed as percentages of the maximal control agonist-induced responses. (c) In additional experiments, aortic rings were incubated in Ca^{2+}-free PSS for 2 h and then washed 3 times in Ca^{2+}-free high-KCl (80 mM) solution for 5 min. At this time the $CaCl_2$ concentration in the bathing media was increased stepwise and concentration-response curves to $CaCl_2$ (0.5–6 mM) were obtained. After washing in Ca^{2+}-free PSS, the preparations were exposed to visnagin for 15 min after which the concentration-response curve to $CaCl_2$ was repeated. The results were expressed as percentages of the control contractile response induced by 6 mM $CaCl_2$. (d) To study the effects of visnagin on an intracellular Ca^{2+} store mobilized by noradrenaline, the experimental protocol was similar to that previously described (Duarte et al., 1994). Aortic rings incubated in normal PSS were initially exposed to KCl (80 mM) until the contractile response reached a steady tension and were then washed in PSS for 5 min. The incubation media were then changed to a Ca^{2+}-free PSS (0.5 mM EGTA) for 15 min and at this time the aortic rings were stimulated by 10^{-5} M noradrenaline for 5 min to produce a typical phasic contraction. Experimental muscles were pretreated for 15 min in the presence of visnagin (10^{-5} M–10^{-4} M) before the addition of the agonists. The responses to agonists were expressed as percentages of the initial contractions induced by 80 mM KCl. (e) Aortic rings incubated in Ca^{2+}-free PSS were contracted by successive additions of 10^{-5} M noradrenaline until the contractile response to this agonist was suppressed. At this time, the rings were contracted with 10^{-7} M PMA and when the contractile response to PMA reached its maximum, visnagin was added in a cumulative fashion. (f) The effects of visnagin on noradrenaline-stimulated Ca^{2+} entry were studied according to the following experimental protocol (Pérez-Vizcaíno et al., 1993). Aortic rings were initially contracted with 10^{-5} M noradrenaline. After washing, rings were incubated in Ca^{2+}-free PSS with 10^{-5} M verapamil for 10 min. At this time, the addition of 10^{-5} M noradrenaline induced a phasic contraction. After 20 min, when the basal tension was reached, the con-

centration of $CaCl_2$ in the bathing media was increased to 2 mM $CaCl_2$ and a tonic contraction was recorded. In experimental muscles visnagin was added 15 min before the addition of $CaCl_2$. Results are expressed as percentages of the initial noradrenaline-induced contractions. (g) To study the effects of visnagin on the spontaneous portal vein contractions, cumulative concentration-response curves were obtained in the absence or presence of glibenclamide. Appropriate parallel control experiments were performed using the same experimental protocols but omitting visnagin.

2.2. Effects on $^{86}Rb^+$ efflux

The effects of visnagin on $^{86}Rb^+$ efflux were determined as described by Pérez-Vizcaíno et al. (1993). Aortic rings were equilibrated for 10 min in a PSS of the following composition (mM): NaCl 140, KCl 4.75, $CaCl_2$ 1.5, $MgSO_4$ 1, glucose 11 and Hepes 10 at pH 7.4 bubbled with 100% O_2 at 37°C. The rings were loaded for 3 h in PSS containing $^{86}Rb^+$ (5 μCi ml^{-1}). Afterwards, the muscles were perfused with PSS at a rate of 1 ml min^{-1} for 40 min to remove excess loosely bound radioactivity. Thereafter, the perfusate was collected at 3 min intervals. Firstly, rings were perfused with PSS for the first 15 min and then with PSS containing visnagin (10^{-4} M or 3×10^{-4} M) or levcromakalin (10^{-6} M). At the end of the experiment, the radioactivity remaining in the aorta was determined by dissolving the vessel in 200 μl of a solution containing equal parts perchloric acid (37% w/v) and H_2O_2 (30 volumes) heated for 15 min at 75°C. The $^{86}Rb^+$ activity in the perfusate and that extracted from the tissues were measured by liquid scintillation counting. The results were expressed in terms of efflux rate constants which reflect the permeability of the cell membrane to Rb^+. Rate constants (k) during each time interval were calculated from the following equation: $k = \ln (A_1/A_2)/(t_2 - t_1)$, where A_1 and A_2 represent the total tissue counts at time points t_1 and t_2, respectively.

2.3. Drugs

The following drugs were used: visnagin (Aldrich Quimica, Madrid), levcromakalin (SmithKline Beecham Pharmaceuticals, Betchworth, UK), noradrenaline bitartrate, glibenclamide, tetraethylammonium chloride, verapamil and phorbol 12-myristate 13-acetate (Sigma Co., London). Visnagin, PMA and glibenclamide were initially dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution and further dilutions were made in PSS. The final DMSO concentrations did not produce significant effects on contractile responses or $^{86}Rb^+$ efflux. Ascorbic acid (10^{-4} M) was added to each solution of noradrenaline, made up freshly every day.

2.4. Statistics

Values are expressed throughout as means ± S.E.M. and statistical analysis was performed with Student's t-test. The differences between control and experimental values were considered significant when $P < 0.05$. Concentration-response slopes were analyzed to give the concentration of visnagin producing a 50% inhibition of the maximal contractile response (IC_{50}) using linear regression analysis over the response range of 20–80% of the maximal inhibition.

3. Results

3.1. Effects on myogenic portal activity

In 14 portal vein segments the control amplitude of spontaneous contractions was 298 ± 42 mg. Visnagin (10^{-6} M–2×10^{-4} M) inhibited the amplitude of these contractions in a concentration-dependent manner ($IC_{50} = 2.0 \pm 0.3 \times 10^{-5}$ M, $n = 7$) and at 2×10^{-4} M it suppressed spontaneous activity. This effect was accompanied by a progressive increase in the rate of spontaneous contractions which was maximal at 7.5×10^{-5} M visnagin (158 ± 15% of control). Pretreatment with the specific inhibitor of ATP-dependent K^+ channels, glibenclamide (10^{-6} M), had no significant effects on visnagin-induced inhibition of spontaneous contractions ($IC_{50} = 1.3 \pm 0.3 \times 10^{-5}$ M, $n = 7$, $P > 0.05$).

3.2. Effects on depolarization-induced contractions in rat aorta

At concentrations between 10^{-6} M and 2×10^{-4} M, visnagin had no effect on baseline tension in aortic rings. Addition of 20 or 80 mM KCl to aortic rings induced a contractile response which averaged 381 ± 24 mg ($n = 7$) and 815 ± 87 mg ($n = 9$), respectively. Pretreatment with visnagin ($> 10^{-6}$ M) inhibited the contractile responses induced by either concentration of KCl in a concentration-dependent manner, the IC_{50}s being $1.7 \pm 0.2 \times 10^{-5}$ M and $8.7 \pm 0.9 \times 10^{-5}$ M, respectively. Thus, visnagin was more potent ($P < 0.05$) to inhibit the contractions induced by mild (20 mM KCl) than by strong (80 mM KCl) depolarizations. These inhibitory effects were reversed after washing for 15 min with drug-free PSS. Fig. 1 shows that cumulatively increased concentrations of visnagin (10^{-6} M–2×10^{-4} M) in aortic rings previously contracted with 30 or 80 mM KCl also resulted in a concentration-dependent relaxation, the IC_{50} values for this effect being $5.4 \pm 0.5 \times 10^{-5}$ M ($n = 7$) and

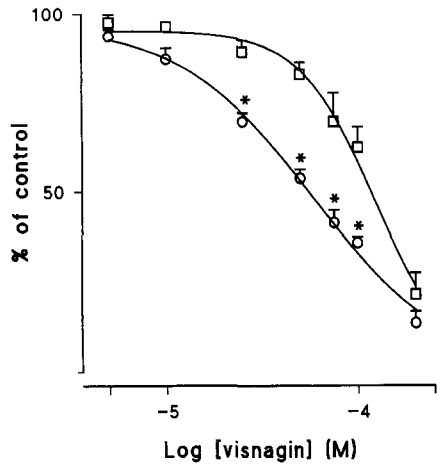

Fig. 1. Effects of visnagin added cumulatively to aortic rings previously contracted with 30 (○) or 80 mM KCl (□). Ordinate scale: percent of control values. Abscissa scale: log visnagin concentration (M). Each point represents the mean ± S.E.M. of six to seven experiments. $^{*}P < 0.05$ vs. 80 mM KCl.

$1.2 \pm 0.2 \times 10^{-4}$ M ($n = 6$), respectively. Thus, visnagin was again more potent to relax contractions induced by mild than by strong depolarization ($P < 0.05$). Pretreatment with glibenclamide (10^{-6} M) did not change the relaxant effects of visnagin on 30 mM KCl-induced contractions (($IC_{50} = 5.5 \pm 0.9 \times 10^{-5}$ M, $n = 6$, $P > 0.05$).

The inhibitory effects of visnagin on the concentration-response curves to $CaCl_2$ are shown in Fig. 2. Following incubation for 5 min in Ca^{2+}-free high-KCl (80 mM) PSS, the cumulative addition of $CaCl_2$ to the bathing media (0.5–6 mM) caused a concentration-dependent contraction of the aortic rings. In six muscles

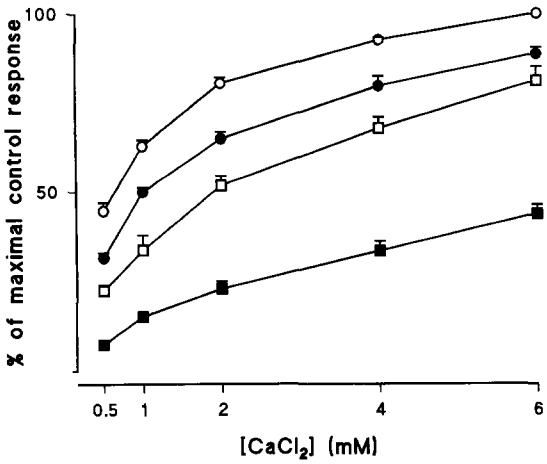

Fig. 2. Effects of visnagin on the aortic contractions induced by addition of $CaCl_2$ (0.5–6 mM) to Ca^{2+}-free high KCl (80 mM) PSS. Ordinate scale: percentage of the maximum control contractions obtained with the highest concentration of $CaCl_2$ in each experiment. Abscissa scale: $CaCl_2$ concentration (mM). Each point represents the mean ± S.E.M. of six experiments. Control (□), after visnagin 5×10^{-5} M (●), 10^{-4} M (□) and 2×10^{-4} M (■).

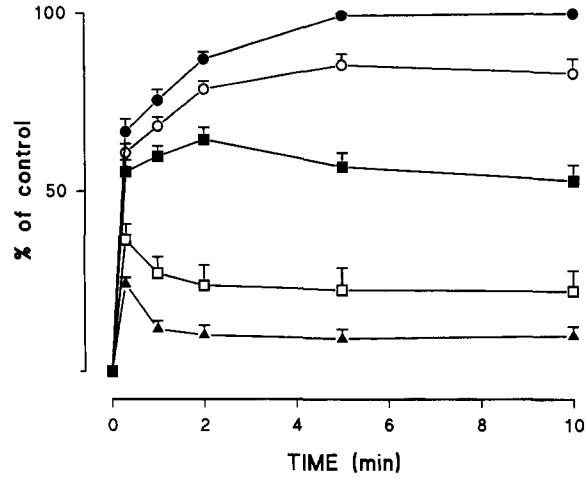

Fig. 3. Time-course of the inhibitory effects of visnagin on the contractile responses induced by noradrenaline (10^{-5} M). Contractions were recorded in the presence of vehicle (●) or in the presence of visnagin 5×10^{-6} M (○), 10^{-5} M (■), 5×10^{-5} M (□) and 10^{-4} M (▲). Ordinate scale: percent of maximal control response. Abscissa scale: time (min). Each point represents the mean ± S.E.M. of seven experiments.

the maximum contractile response induced by 6 mM $CaCl_2$ was 643 ± 70 mg. Visnagin, 5×10^{-5} M, 10^{-4} M and 2×10^{-4} M, produced a concentration-dependent decrease in the amplitude of $CaCl_2$-induced contractions of KCl-depolarized aortic rings and caused a progressive shift of the $CaCl_2$ concentration-response curves downwards, so that the IC_{50} calculated at 6 mM $CaCl_2$ was $1.8 \pm 0.1 \times 10^{-4}$ M.

3.3. Effects on noradrenaline-induced contractions in rat aorta in normal PSS

Addition of 10^{-5} M noradrenaline to the bathing media induced a contraction of rat aortic rings averaging 833 ± 60 mg ($n = 8$). Fig. 3 shows that noradrenaline-induced contractions are characterized by a phasic and a tonic component and visnagin markedly changed the time-course of these contractions. Visnagin 5×10^{-6} M–10^{-4} M decreased both components, but at concentrations higher than 10^{-5} M the contractile response displayed a rapid phasic contraction which quickly relaxed and reached a stable tension within 2 min. The IC_{50} values to inhibit the phasic and tonic components of noradrenaline-induced contractions were $5.9 \pm 0.3 \times 10^{-5}$ M and $1.4 \pm 0.3 \times 10^{-5}$ M, respectively, which indicated that visnagin ($> 5 \times 10^{-6}$ M) inhibited the tonic component of the contraction to a greater extent ($P < 0.05$).

Cumulative increases in the concentration of visnagin (10^{-6} M–2×10^{-4} M) in aortic rings previously contracted with 10^{-5} M noradrenaline, also resulted in a concentration-dependent relaxation, the IC_{50} values for this effect were $2.2 \pm 0.4 \times 10^{-5}$ M. The relaxant

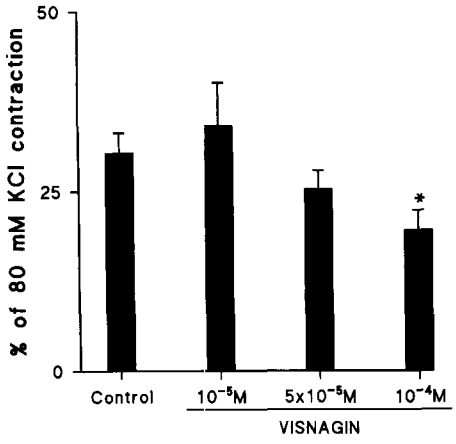

Fig. 4. Effects of visnagin on the phasic contractions induced by noradrenaline in aortic rings incubated in Ca^{2+}-free 0.5 mM EGTA. Visnagin (10^{-5} M–10^{-4} M) was added 15 min before production of the phasic contraction with 10^{-5} M noradrenaline. Ordinate scale: percent of the control response to 80 mM KCl. Each bar represents the mean ± S.E.M. of seven to nine experiments. $^{*}P < 0.05$ vs. control.

effects on noradrenaline-induced contractions were not altered in the absence of functional endothelium ($IC_{50} = 3.0 \pm 0.4 \times 10^{-5}$ M. $n = 7$, $P > 0.05$) when compared to endothelium-intact rings). Furthermore, the relaxant response to visnagin on noradrenaline-induced contractions was not affected by pretreatment with 30 mM tetraethylammonium ($n = 3$, not shown).

In another group of experiments, the muscles were first exposed to 80 mM KCl and when the contractile response reached steady-state, 10^{-5} M noradrenaline was added to the bathing media. Under these conditions, visnagin was less potent to inhibit noradrenaline-induced contractions ($IC_{50} = 8.1 \pm 1.7 \times 10^{-5}$ M, $n = 7$, $P < 0.05$).

3.4. Effects on noradrenaline-induced contractions in Ca^{2+}-free PSS and after restoring extracellular Ca^{2+}

To study the effects of visnagin on the phasic contractile responses induced by noradrenaline, aortic rings were incubated in Ca^{2+}-free PSS containing 0.5 mM EGTA. The addition of 10^{-5} M noradrenaline resulted in a phasic contraction which averaged 30.4 ± 2.8% ($n = 9$) of the initial contraction induced by 80 mM KCl. Fig. 4 shows that visnagin only produced a significant inhibition ($P < 0.05$) of this phasic contractile response at a concentration of 10^{-4} M.

In another group of experiments, the effects of visnagin were studied on the contractile responses induced by restoration of extracellular Ca^{2+} in aortic rings incubated in Ca^{2+}-free PSS containing 10^{-5} M verapamil. Under these conditions, 10^{-5} M noradrenaline induced a phasic contraction. After 20 min, 2 mM $CaCl_2$ was added to the bathing media resulting in a tonic contractile response which averaged 79.6 ± 6.0% of an initial contraction to noradrenaline in normal PSS. As shown in Fig. 5, visnagin (5×10^{-6} M–2×10^{-4} M), produced concentration-dependent inhibition of the tonic contractions induced by noradrenaline under these experimental conditions, with significant differences ($P < 0.05$) at concentrations $> 5 \times 10^{-6}$ M ($IC_{50} = 4.4 \pm 0.1 \times 10^{-5}$ M).

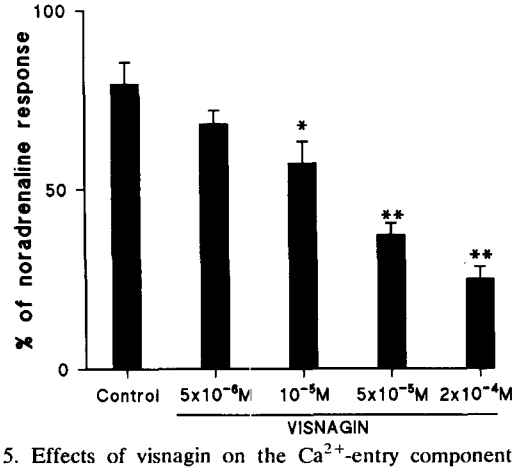

Fig. 5. Effects of visnagin on the Ca^{2+}-entry component of noradrenaline-induced contractions. A phasic contraction was initially elicited with 10^{-5} M noradrenaline in Ca^{2+}-free PSS supplemented with 10^{-5} M verapamil. 5 min later visnagin was added for 15 min and finally a sustained contraction was induced by restoring extracellular Ca^{2+} (2 mM $CaCl_2$). Ordinate scale: percent of the control response induced by 10^{-5} M noradrenaline in PSS. Each bar represents the mean ± S.E.M. of 7–12 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

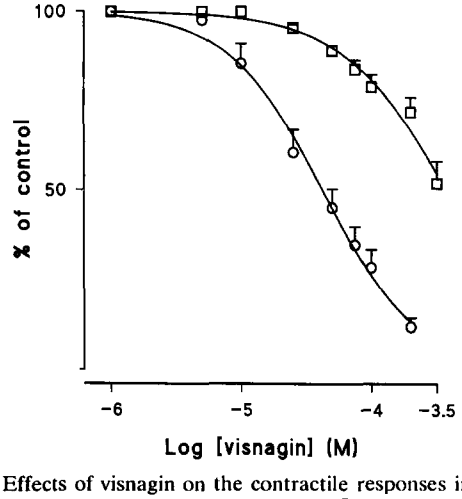

Fig. 6. Effects of visnagin on the contractile responses induced with phorbol 12-myristate 13-acetate (PMA, 10^{-7} M) in the presence (○) or in the absence (□) of extracellular Ca^{2+}. The contractions due to PMA in the absence of extracellular Ca^{2+} were elicited after depletion of intracellular noradrenaline-sensitive Ca^{2+} stores by successive additions of noradrenaline 10^{-5} M for 5 min until no response could be elicited. Ordinate scale: percent of control values. Abscissa scale: log visnagin concentration (M). Each point represents the mean ± S.E.M. of 7–12 experiments.

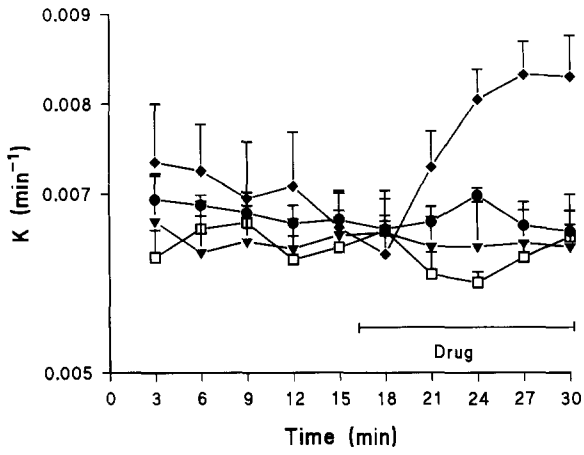

Fig. 7. Effects of visnagin on the $^{86}Rb^+$ efflux in non-stimulated rat aortic rings. $^{86}Rb^+$ loaded rings were placed in PSS for 40 min to remove loosely bound $^{86}Rb^+$. Then, rings were perfused for 15 min with PSS and, thereafter, in PSS containing vehicle (●), 10^{-5} M levcromakalim (◆), 10^{-4} M visnagin (▼) or 3×10^{-4} M visnagin (□). Ordinate scale: efflux rate constant (min^{-1}). Abscissa scale: time (min). Each point represents the mean ± S.E.M. of five to seven experiments.

3.5. Effects on PMA-induced contractions

PMA (10^{-7} M) produced a sustained contraction of aortic rings incubated in either PSS (512 ± 105 mg, $n = 8$) or in Ca^{2+}-free PSS after complete depletion of noradrenaline-sensitive intracellular Ca^{2+} stores by successive additions of 10^{-5} M noradrenaline until no contractile responses to this agonist could be elicited (382 ± 47 mg, $n = 7$). As shown in Fig. 6, visnagin, 5×10^{-6} M–5×10^{-4} M, when added at the plateau of PMA-induced contractions, produced a concentration-dependent relaxation of aortic rings incubated in PSS ($IC_{50} = 4.3 \pm 0.8 \times 10^{-5}$ M) and with a lower potency in Ca^{2+}-free PSS ($IC_{50} \geq 5\times10^{-4}$ M, $P < 0.01$ vs. Ca^{2+}-containing PSS).

3.6. Effects on $^{86}Rb^+$ efflux

The effects of visnagin were studied on $^{86}Rb^+$ efflux. As shown in Fig. 7, visnagin (10^{-4} M and 2×10^{-4} M), had no effect on $^{86}Rb^+$ efflux. In contrast, levcromakalim (10^{-6} M), a known potassium channel opener, significantly increased the efflux of $^{86}Rb^+$.

4. Discussion

The present results demonstrated that visnagin inhibited the contractile responses induced in rat aortic rings by: (a) high KCl or increases of extracellular Ca^{2+} in KCl-depolarized aortic rings and (b) noradrenaline and PMA both in a Ca^{2+}-containing or Ca^{2+}-free medium. Furthermore, the relaxation induced by visnagin in aorta precontracted with noradrenaline was not affected by endothelial removal, which indicated that its vasodilator effect is unrelated to the release of endothelium-derived factors. Additionally, visnagin inhibited the spontaneous myogenic contractions of portal veins.

The contractile responses induced by high KCl (80 mM) or increases of $CaCl_2$ in KCl-depolarized muscles are due to the influx of extracellular Ca^{2+} through L-type voltage-sensitive channels (VOCs, Godfraind et al., 1986) and have been used to provide a simple means of studying drugs with possible Ca^{2+}-entry blocking properties (Cauvin et al., 1983; Godfraind et al., 1986; Tamargo and Tejerina, 1989). Visnagin inhibited these contractile responses in a concentration-dependent manner, which suggested that it inhibited Ca^{2+} through VOCs. However, visnagin inhibited the contractions induced by low concentrations (20–30 mM) more effectively than those induced by high concentrations (80 mM) of KCl. Furthermore, visnagin was 4 times less potent to relax noradrenaline-induced contractions in aorta previously depolarized by 80 mM KCl than those induced by noradrenaline in the absence of depolarization. A similar behavior was found with KCl channel openers (Edwards and Weston, 1993; Hamilton and Weston, 1989; Pérez-Vizcaíno et al., 1993). Since visnagin shows some structural similarity with KCl channel openers of the benzopyrane group, we studied whether the vasodilator effect of visnagin could be related to the opening of ATP-sensitive K^+ channels. However, pretreatment with the selective blocker of ATP-dependent K^+ channels, glibenclamide (Ashcroft, 1988), or the non-specific K^+ channel blocker, tetraethylammonium, did not modify visnagin-induced vasorelaxation. Furthermore, visnagin did not increase $^{86}Rb^+$ efflux, a substitute cation for K^+. Thus, it is unlikely that the vasodilator effect of visnagin can be related to the opening of ATP-sensitive K^+ channels.

The portal vein develops spontaneous rhythmic transient membrane depolarizations coupled to Ca^{2+}-dependent contractions. This myogenic activity can be inhibited by removal of external Ca^{2+}, Ca^{2+} channel blockers and K^+ channel openers, suggesting that myogenic activity depends on Ca^{2+} influx probably through VOCs (Cauvin et al., 1983; Dacquet et al., 1987). Visnagin inhibited the spontaneous contractions in a concentration-dependent manner and the IC_{50} values for inhibition of myogenic activity and the contractile responses induced by 20 mM KCl were quite similar. Moreover, the inhibition of spontaneous activity persisted after pretreatment with glibenclamide, which again suggested that its inhibitory effect is not mediated by activation of ATP-dependent K^+ channels but may be explained by its ability to decrease Ca^{2+} through VOCs.

In rat aorta, contractions induced by activation of α_1-adrenoceptors can be resolved into a phasic and a tonic component (Hudgins and Weiss, 1968; Godfraind and Kaba, 1969). The phasic component has been attributed to IP_3-mediated release of Ca^{2+} from intracellular stores (Chiu et al., 1987), whereas the tonic component is related to an increase in extracellular Ca^{2+} entry via pathways sensitive and insensitive to Ca^{2+}-entry blockers (Rüegg et al., 1989; Cauvin and Malik, 1984). Visnagin not only inhibited both components but markedly changed the time-course of noradrenaline-induced contractions, so that at concentrations $> 10^{-5}$ M, the phasic component was followed by a relaxation phase and a steady-state contractile response was reached within 2 min. This time-course was similar to that in the absence of extracellular Ca^{2+} and suggested that visnagin inhibited the tonic component to a greater extent by reducing noradrenaline-induced Ca^{2+} entry. To further analyze the effects of visnagin on both components two experiments were performed. First, we studied the effects of visnagin on the phasic contraction induced by noradrenaline in Ca^{2+}-free PSS which has been attributed to the release of Ca^{2+} from a noradrenaline-sensitive intracellular store (Cauvin et al., 1983; Godfraind et al., 1986; Tamargo and Tejerina, 1989). Second, the effects of visnagin were studied on the sustained contraction elicited following the addition of $CaCl_2$ to a Ca^{2+}-free PSS containing a high concentration of verapamil in the presence of noradrenaline. Under these conditions, Ca^{2+} was added after the phasic contraction induced by noradrenaline had developed; the entry of Ca^{2+} through VOCs is blocked by verapamil and the intracellular stores cannot be refilled in the continuous presence of noradrenaline (Karaki et al., 1979). Therefore, these responses most likely represent an index of Ca^{2+} entry through specific noradrenaline-activated, verapamil-insensitive, channels. Comparing the results of both designs made it evident that visnagin was almost 10 times more potent to inhibit the sustained (Fig. 5) compared to the phasic contractile response (Fig. 4) induced by noradrenaline. Thus, visnagin preferentially inhibited the component of noradrenaline-induced contractions due to Ca^{2+} entry via pathways insensitive to Ca^{2+}-entry blockers. Moreover, these results confirmed that visnagin seems to be more potent to inhibit Ca^{2+} entry through L-type and receptor-activated channels than the release of Ca^{2+} from intracellular stores. Another possible explanation for the present results is that visnagin enhances Ca^{2+} extrusion from vascular smooth muscle cells, a phenomenon not studied in the present experiments.

Protein kinase C plays an important role in the tonic contraction of vascular smooth muscle (Andrea and Walsh, 1992). Tumor-promoting phorbol esters, like PMA, activate protein kinase C and induce a sustained contractile response in rat aorta which can be attributed to both an increase in Ca^{2+} entry and an increase in Ca^{2+} sensitivity of contractile proteins (Sato et al., 1992). In our study, PMA induced contractile responses, even in Ca^{2+}-free media and after depletion of noradrenaline-sensitive intracellular Ca^{2+} stores, which were lower than PMA-induced responses in the presence of extracellular Ca^{2+}. Since PMA-induced responses in Ca^{2+}-free media occur without significant changes in $[Ca^{2+}]_i$ they have been attributed to increased Ca^{2+} sensitivity of myofilaments (Sato et al., 1992). These responses were inhibited by visnagin at concentrations higher than those needed in the presence of extracellular Ca^{2+}, suggesting that visnagin preferentially inhibited the component of PMA-induced contractions due to Ca^{2+} entry.

The vasodilator profile of visnagin seems to be quite similar to that previously reported for its methylated analog, khellin (Ubeda and Villar, 1989; Ubeda et al., 1991). However, khellin was less potent to inhibit noradrenaline-induced contractions ($IC_{50} = 2.3 \times 10^{-4}$M, Ubeda et al., 1991) than visnagin ($IC_{50} = 2.2 \times 10^{-5}$M, present results) whereas the potency of both drugs was similar on 80 mM KCl-induced contractions (IC_{50}s = 1.8×10^{-4} M and 1.2×10^{-4} M, respectively). At concentrations $> 10^{-4}$ M khellin and visnagin inhibited the phasic contractions induced by noradrenaline or caffeine in Ca^{2+}-free media.

In conclusion, the results from this study showed that visnagin inhibited vascular smooth muscle contractility by acting at multiple sites. In the range of 10^{-6} M to 5×10^{-5} M visnagin appears to inhibit only the contractions mediated by Ca^{2+} entry through pathways with low sensitivity to classical Ca^{2+}-entry blockers, i.e. agonist-, PMA- or mild depolarization-induced Ca^{2+} entry. Therefore, the vasodilator profile of visnagin is not that of typical Ca^{2+}-entry blockers which preferentially inhibit the contractions induced by strong depolarizations. At higher concentrations ($> 5 \times 10^{-5}$ M) visnagin causes non-specific inhibition of vascular smooth muscle contractility.

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