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Vasorelaxation of Rat Thoracic Aorta Caused by Two Ca^{2+}-Channel Blockers, HA-22 and HA-23

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Abstract—The pharmacological effects of HA-22 (2-(4′methoxyphenylmethyl)-3,4-dimethylpyrano[2,3-c]pyrazol-6(2H)-one) and HA-23 (2-(2′-thienylmethyl)-3,4-dimethylpyrano[2,3-c]pyrazol-6(2H)-one) on rat isolated thoracic aorta have been examined. In high potassium medium (60 mM), Ca^{2+} (0·03–3 mM)-induced vasoconstriction was inhibited by HA-22 and HA-23 (10–100 $\mu g\ mL^{-1}$). Cromakalim-relaxed aortic rings precontracted with 15 mM but not 60 mM K^+. However, HA-22, HA-23 and verapamil produced a greater relaxation in 60 mM than in 15 mM K^+-induced contraction. The tonic contractions elicited by KCl (60 mM) and Bay K 8644 (10^{-7} M) were also relaxed by the addition of HA-22 and HA-23. The phenylephrine concentration-response curves displayed antagonism by HA-22 and HA-23 (10–100 $\mu g\ mL^{-1}$) in a non-competitive manner. The caffeine (10 mM)-induced contraction and cAMP or cGMP levels were not affected by HA-22 or HA-23. It is concluded that HA-22 and HA-23 relaxed the rat aorta by suppressing the Ca^{2+} influx through both voltage-dependent and receptor-operated Ca^{2+} channels.

Ca^{2+}-channel blocker has become a broader term for drugs which lower cytosolic Ca^{2+} concentration by inhibiting Ca^{2+} entry into the cell. It is clear that a Ca^{2+}-channel blocker may have various mechanisms and sites of action (Triggle & Swamy 1983; Rampe et al 1985). For example, pinacidil is a new antihypertensive agent, which may relax vascular smooth muscle (Cohen & Colbert 1986). The mechanisms of action are thought to be due to inhibition of Ca^{2+} influx in K^+-contracted arterial strips (Videbaek et al 1988; Masuzawa et al 1990) and opening of K^+ channels in the smooth muscle, thus increasing the K^+-permeability and hyperpolarizing the cell membrane (Cook et al 1988).

In a large scale screening test, we have found many compounds inhibited the contraction of aortic smooth muscles. For example, magnolol causes vasorelaxation of rat aorta by releasing endothelium-derived relaxing factor (EDRF) (Teng et al 1990a); two vasorelaxants, denudatin B and fargesone B block the voltage-dependent Ca^{2+} channel (Teng et al 1990b; Yu et al 1990); norathyriol is also a Ca^{2+}-channel blocker (Ko et al 1991).

Recently, we found HA-22 (2-(4′-methoxyphenylmethyl)-3,4-dimethylpyrano [2,3-c]pyrazol-6(2H)-one) and HA-23 (2-(2′-thienylmethyl)-3,4-dimethylpyrano [2,3-c] pyrazol-6(2H)-one) (Fig. 1), possessed vasorelaxing action in rat thoracic aorta. We now describe these effects of HA-22 and HA-23 and attempt to characterize their modes of action.

Materials and Methods

Mechanical response

Wistar rats of either sex, 250–300 g, were killed by a blow to the head. The thoracic aorta was isolated and excess fat and connective tissue were removed. The vessels were cut into rings of about 5 mm in length and mounted in organ baths containing 5 mL of Krebs solution of the following composition (mM): NaCl 118·2, KCl 4·7, $CaCl_2$ 1·9, $MgSO_4$ 1·2, KH_2PO_4 1·2, $NaHCO_3$ 25 and glucose 11·7. The tissue bath solution was maintained at 37°C and bubbled with 95% O_2–5% CO_2. Two stainless steel hooks were inserted into the aortic lumen, one was fixed while the other was connected to a transducer. The aortae were equilibrated in the medium for 90 min with three changes of Krebs solution and maintained under an optimal tension of 1 g before specific experimental protocols were initiated. Contractions were recorded isometrically via a force-displacement transducer connected to a Gould polygraph (Model 2400). In all experiments, the endothelium was removed by rubbing with a cotton ball, and the absence of acetylcholine (3 μM)-induced relaxation was taken as an indicator that vessels were denuded successfully.

The contractile effect of calcium was studied in rings stabilized in high K^+ solution without Ca^{2+}. Calcium was then added from stock dilution to obtain the desired concentrations, and the effect of each Ca^{2+} concentration was recorded. The maximal tension attained at 3 mM Ca^{2+} was considered as 100%. The high K^+ solution was prepared by substituting NaCl with KCl (15 or 60 mM) in an equimolar amount. An IC50 for the inhibitory action of HA-22 and HA-23 was calculated as the concentration of either agent which reduced the tension to 50% of 1 mM Ca^{2+}-induced tension in the control of the same vessel.

cAMP and cGMP assay of rat aorta

The content of cAMP and cGMP in aorta was assayed as described previously (Itoh et al 1982; Kauffman et al 1987). After incubation of aortic rings with dimethylsulphoxide, forskolin, sodium nitroprusside, HA-22 or HA-23 for 2 min, the aortic rings were rapidly frozen in liquid nitrogen and stored at −80°C until homogenized in 0·5 mL of 10% trichloroacetic acid using a Potter glass/glass homogenizer. The homogenate was centrifuged at 10000 *g* for 5 min and the supernatant was removed and extracted with 4 × 3 vol of ether, and the cAMP or cGMP content was then assayed

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using RIA kits. The precipitate was used for protein assay (Lowry et al 1951). cAMP and cGMP levels were expressed as pmol (mg protein)$^{-1}$.

Materials

Phenylephrine, 3-isobutyl-1-methyl-xanthine (IBMX), sodium nitroprusside, forskolin, verapamil, nifedipine, Bay K 8644 and caffeine were obtained from Sigma Chemical Co. cAMP and cGMP RIA kits were purchased from Amersham. If drugs were dissolved in dimethylsulphoxide (DMSO), the final concentration of DMSO in the bathing solution did not exceed 0·1% and had no affect on the muscle contraction. All experiments with Bay K 8644, verapamil and nifedipine were conducted in the dark.

Data analysis

The experimental results are expressed as the mean ± s.e. and accompanied by the number of observations. Statistical significance was evaluated by Student's *t*-test and *P* values less than 0·05 were considered to be significant.

Results

Effects of HA-22 and HA-23 on high K^+-induced calcium-dependent contraction

In Ca^{2+}-free Krebs solution containing high K^+ (60 mM), the cumulative addition of Ca^{2+} (0·03–3 mM) caused a stepwise increase of contraction force. After pretreatment of aorta with HA-22 or HA-23 (10–100 μg mL^{-1}) for 15 min, both inhibited this Ca^{2+} contraction in a concentration-dependent manner (Fig. 1). The IC50 values for HA-22 and HA-23 were calculated to be about 8·6 and 8·9 μg mL^{-1}, respectively (for a calcium concentration of 1 mM).

Relaxant responses to HA-22, HA-23, cromakalim and verapamil

Relaxant responses to HA-22, HA-23, cromakalim and verapamil were compared in aortic rings precontracted with 15 or 60 mM K^+ (Fig. 2A–D). Cromakalim relaxed aortic rings precontracted with 15 mM but not 60 mM K^+ (Fig. 2C). However, HA-22, HA-23 or verapamil produced a greater relaxation in 60 mM than in 15 mM K^+-induced contractions (Fig. 2A, B, D).

Effects of HA-22, HA-23 and nifedipine on KCl- and Bay K 8644-induced tonic contractions

Exposure of rat aorta to KCl (60 mM) and Bay K 8644 (10^{-7} M) caused a tonic contraction maintained at least for 25 min. If HA-22, HA-23 (100 μg mL^{-1}) or nifedipine (1 μM) was added during tonic contraction (10 min after the exposure to KCl or Bay K 8644), the relaxation could be observed (Fig. 3A, B).

Effects of HA-22, HA-23 and nifedipine on phenylephrine-induced contraction

Cumulative addition of phenylephrine (10^{-8} – 3×10^{-5} M) to Krebs medium caused a stepwise increase of tension of the rat aorta. After pretreatment of aorta with HA-22 and HA-23 (10–100 μg mL^{-1}), both produced a non-competitive blockade (Fig. 4A, B). Phenylephrine (3 μM) caused a phasic and then a tonic contraction maintained for at least 30 min.

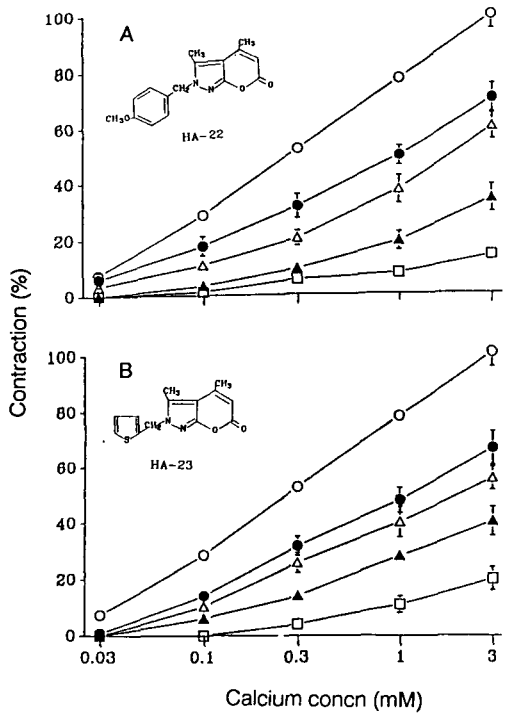

FIG. 1. Effects of HA-22 (A) and HA-23 (B) on the Ca^{2+}-dependent contraction of rat aorta induced by high K^+. In the medium of high K^+ (60 mM), various concentrations of HA-22 and HA-23 (○ control, ● 10, △ 20, ▲ 50, □ 100 μg mL^{-1}) were pre-incubated with aorta at 37°C for 15 min, then cumulative concentrations of Ca^{2+} (0·03–3 mM) were used to trigger the contraction. The values expressed as the % of control were presented as the mean ± s.e. of four determinations.

HA-22 and HA-23 also caused the relaxation of tonic contraction induced by phenylephrine (data not shown).

Effects of HA-22 and HA-23 on caffeine-induced contraction

Caffeine (10 mM) caused a rapid phasic contraction (0·48 ± 0·02 g, n = 4). HA-22, HA-23 (100 μg mL^{-1}) and nifedipine (2 μM) did not affect this contraction, while procaine (10 mM) and, cromakalim (3 μM) markedly inhibited the caffeine-induced contraction (Fig. 5).

Effects of HA-22 and HA-23 on cAMP and cGMP formation

The cyclic nucleotide content of aorta was measured by RIA. The levels of cAMP and cGMP in unstimulated muscles were very low (4·03 ± 0·29, 2·17 ± 0·12 pmol (mg protein)$^{-1}$, respectively. Forskolin (1 μM) and sodium nitroprusside (10 μM) increased the cAMP and cGMP levels to 7·43 ± 0·27 and 5·60 ± 0·6, respectively. However, neither the cAMP nor the cGMP levels were changed by HA-22 or HA-23 (Table 1).

Discussion

It is widely accepted that contraction of vascular smooth muscle requires the increase of cytosolic free Ca^{2+}. The high K^+-induced contraction of smooth muscle is the result of an increase in Ca^{2+} influx through voltage-dependent Ca^{2+}

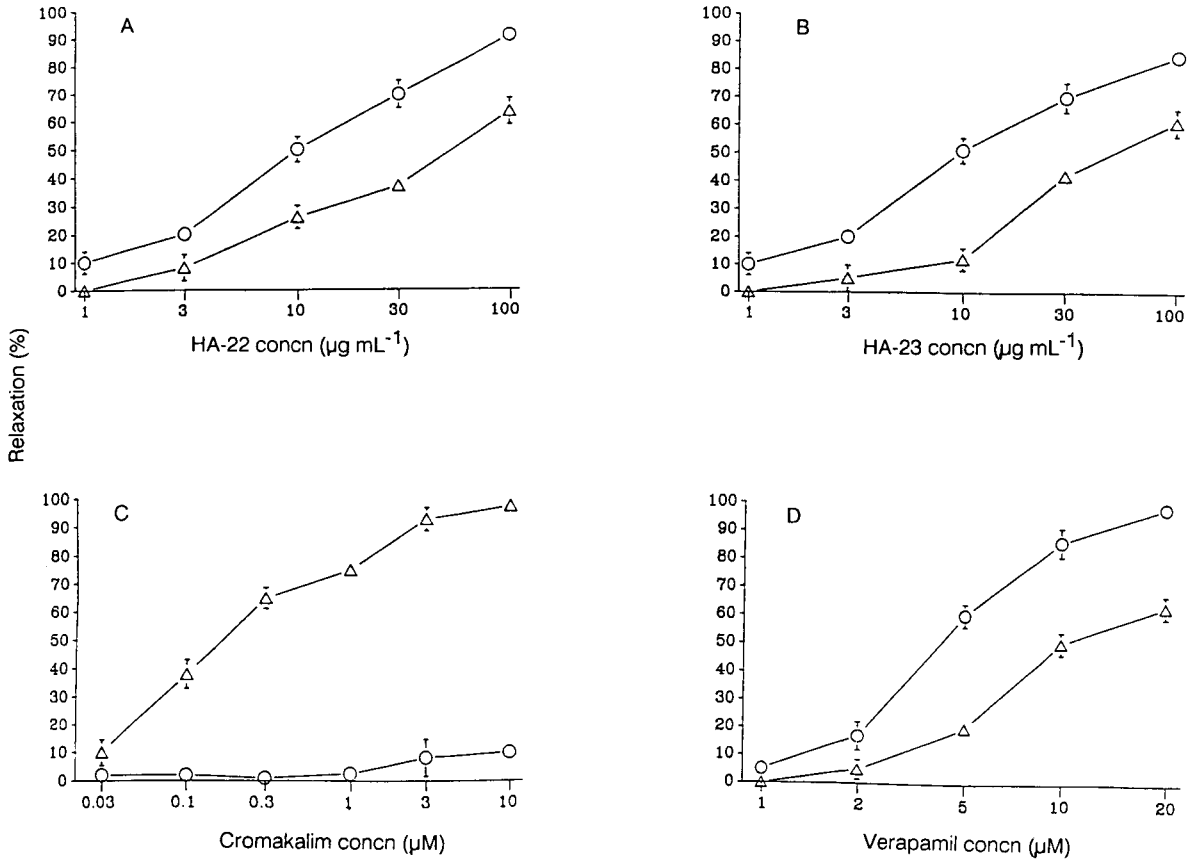

FIG. 2. Rat aortic rings precontracted with 15 (△) or 60 (○) mM K^+: concentration-response curves for the relaxant actions of HA-22 (A), HA-23 (B), cromakalim (C) and verapamil (D).

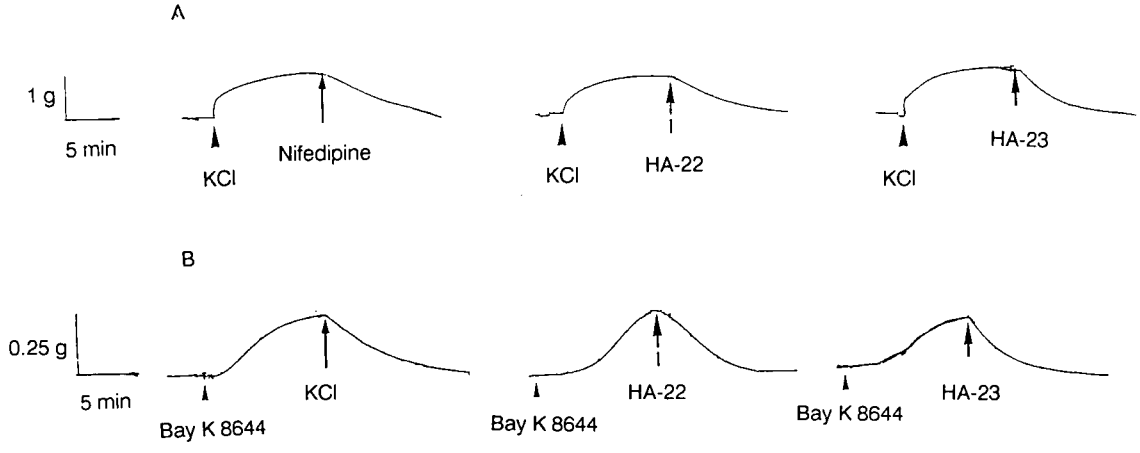

FIG. 3. Typical tracings of HA-22 and HA-23 inhibition on the KCl- and Bay K 8644-induced contractions of rat aorta. Exposure of aortic rings to KCl (60 mM, A) or Bay K 8644 (0·1 μM, B) caused tonic contraction maintained for 10 min, then nifedipine (1 μM), HA-22 (100 μg mL^{-1}) or HA-23 (100 μg mL^{-1}) was added.

channels (Karaki & Weiss 1979, 1984). Bay K 8644 promoted Ca^{2+} influx also through those in vascular smooth muscle (Franckowiak et al 1985; Stash & Kazda 1989). HA-22 and HA-23 (10–100 μg mL^{-1}) inhibited high K^+- and Bay K 8644-induced contraction (Figs 1, 3), thus they may be blockers of voltage-dependent Ca^{2+} channels. In vascular tissues, cromakalim inhibited contractions elicited by low concentrations of K^+ (15 mM) but was ineffective against high concentrations of K^+ (60 mM) (Fig. 2C). The mechanism of this effect is related to opening or activation of K^+ channels leading to an increase in the outward K^+ current and thus to cellular hyperpolarization (Hamilton et al 1986; Weir & Weston 1986). HA-22, HA-23 and verapamil produced greater relaxation in 60 mM than in 15 mM K^+-

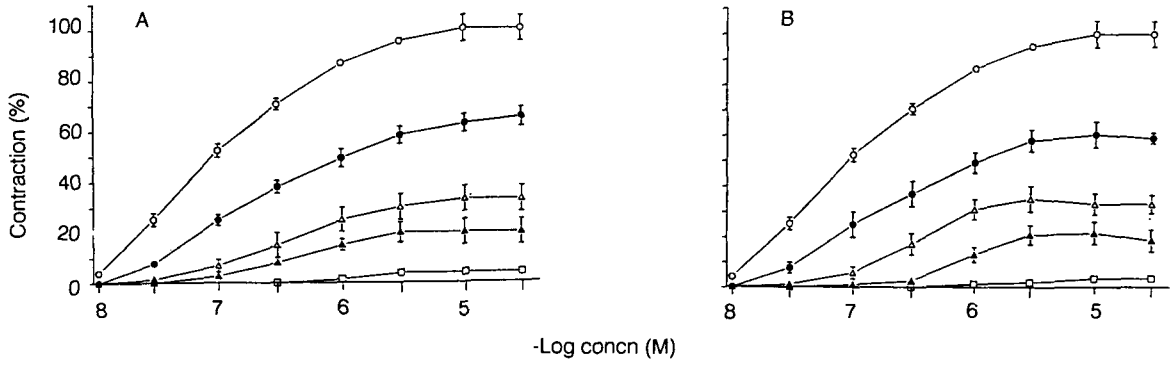

FIG. 4. Antagonism of the concentration-response curves to phenylephrine by 15 min pretreatment of rat aorta with HA-22 (A) and HA-23 (B) (○ control, ● 10 $\mu g\ mL^{-1}$, △ 20 $\mu g\ mL^{-1}$, ▲ 50 $\mu g\ mL^{-1}$, □ 100 $\mu g\ mL^{-1}$). Each point represents the mean and vertical lines show mean ± s.e. (n = 4–6).

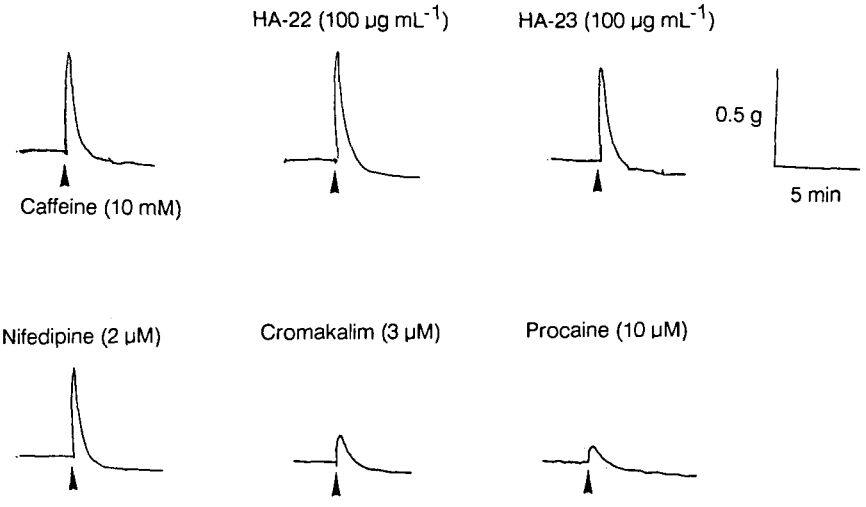

FIG. 5. Typical tracings of the contractile response to caffeine in Ca^{2+}-free Krebs solution. Rat aortic rings were pre-incubated with 0·1% dimethylsulphoxide (control), HA-22 (100 $\mu g\ mL^{-1}$), HA-23 (100 $\mu g\ mL^{-1}$), nifedipine (2 μM), cromakalim (3 μM) and procaine (10 μM) at 37°C for 15 min; caffeine (10 mM) was then used to trigger the phasic contraction.

Table 1. Effects of HA-22 and HA-23 on cGMP and cAMP formation in rat thoracic aorta.

Treatment	cGMP (pmol (mg protein)$^{-1}$)	cAMP (pmol (mg protein)$^{-1}$)
Control	2·17 ± 0·12	4·03 ± 0·29
Sodium nitroprusside (10 μM)	5·60 ± 0·60*	—
Forskolin (1 μM)	—	7·43 ± 0·27*
HA-22 ($\mu g\ mL^{-1}$)		
20	2·34 ± 0·08	3·56 ± 0·35
50	2·11 ± 0·26	4·32 ± 0·03
100	2·27 ± 0·06	4·30 ± 0·25
HA-23 ($\mu g\ mL^{-1}$)		
20	2·32 ± 0·15	3·36 ± 0·25
50	2·39 ± 0·10	4·31 ± 0·47
100	2·23 ± 0·20	3·70 ± 0·34

After preincubation of aortic rings with dimethylsulphoxide (0·1%, control), sodium nitroprusside, forskolin or various concentrations of HA-22, HA-23 for 2 min, the reaction was stopped by immersing the tissue into liquid nitrogen. The cGMP and cAMP contents in rat aorta were measured. The results are expressed as the mean ± s.e. (n = 4).

* $P < 0·001$ compared with the respective control.

induced contraction. This result indicates that the action of HA-22 or HA-23 was mainly due to their Ca^{2+} channel blocking property and was not related to opening of ATP-sensitive K^+ channels.

The increase of tension in response to phenylephrine or noradrenaline was primarily dependent upon Ca^{2+} entry through receptor-operated Ca^{2+} channels and is less dependent upon voltage-dependent Ca^{2+} channel opening (Bolton 1979). Addition of nifedipine eliminates the influence of voltage-dependent Ca^{2+} entry, which would follow noradrenaline-induced membrane depolarization (Mekata 1974,1979). HA-22 and HA-23 still relaxed the noradrenaline-induced membrane depolarization (Mekata 1974, 1979). HA-22 and HA-23 still relaxed the noradrenaline-induced tonic contraction concentration-dependently, in the presence of nifedipine (1 μM), which completely blocked high K^+ (60 mM)-induced contractions. Both agents also caused the relaxation of tonic contraction induced by phenylephine and after pretreatment of aorta with either agent produced the antagonism of the concentration-response curve of phenylephrine in a non-competitive manner. This indicates

that HA-22 and HA-23 also block the Ca^{2+} influx through receptor-operated Ca^{2+} channels.

It is now generally accepted that caffeine can release intracellular Ca^{2+} in vascular smooth muscle (Saida & Van Breemen 1984). However, neither HA-22, HA-23 nor nifedipine affected this process. Cyclic nucleotides have an important role in the relaxation of vascular smooth muscle (Murad 1986). Sodium nitroprusside has been shown to be a potent relaxing agent in vascular smooth muscle. It produces an increase in the level of cGMP via direct activation of guanylate cyclase (Gruetter et al 1979). Forskolin can produce an increase in cAMP levels via activation of adenylate cyclase (Ousterhout & Sperelakis 1987). Neither cAMP nor cGMP content was changed by HA-22 or HA-23 (Table 1). This indicates that the inhibitor effects of HA-22 and HA-23 on the contractile responses caused by high K^+ or phenylephrine are not due to the increase in cyclic nucleotides.

In conclusion, the present results suggest that HA-22 and HA-23 relaxed the rat aorta by suppressing the Ca^{2+} influx through both voltage-dependent and receptor-operated Ca^{2+} channels.

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