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VASORELAXANT ACTION OF CAROVERINE FUMARATE (A QUINOXALINE DERIVATIVE), A CALCIUM-BLOCKING AGENT

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1 Caroverine fumarate, 1-(2-diethylaminoethyl)-3-(*p*-methoxy-benzyl)-1,2-dihydro-2-quinoxalinone fumarate, caused a greater inhibition of the pressor response to KCl (8×10^{-2} M) than that to noradrenaline (10^{-6} M) in the rat hindquarter preparation.

2 In the isolated aorta of the rat, caroverine (up to 10^{-6} M) markedly suppressed the contraction caused by KCl (4×10^{-2} M) (high-K) but had little effect on the contractile response to noradrenaline (10^{-6} M) whether added before the spasmogen or in its presence.

3 In the high-K-treated aorta, caroverine shifted the concentration-response curve for external calcium to the right, competitively. The negative logarithm of the affinity (pA_2) of caroverine was calculated to be approx. 7.

4 Increased ^{45}Ca uptake of the high-K-treated aorta measured by a modified lanthanum method was inhibited by either caroverine (3×10^{-6} M) or verapamil (10^{-6} M).

5 Concentrations of caroverine and verapamil reducing high-K-induced aortic contraction to 50% of its maximum were 2.4×10^{-7} and 6.6×10^{-8} M respectively.

6 Following washout the caroverine-induced inhibition of high-K-induced aortic contraction was more rapidly restored than the verapamil-induced inhibition.

7 These results suggest that caroverine fumarate is a specific and readily reversible calcium influx inhibitor in the rat vascular smooth muscle.

Introduction

Caroverine fumarate, 1-(2-diethylaminoethyl)3-(*p*-methoxy-benzyl)-1,2-dihydro-2-quinoxalinone fumarate, has been reported to have spasmolytic, broncholytic and hypotensive actions (Hornykiewicz, Hitzenberger & Zellner, 1963). Caroverine is being used clinically as a spasmolytic in the form of base or hydrochloride salt. More recently, Oh & Widauer (1977) found that caroverine fumarate caused vasodilatation of the canine cerebral artery. Although Hornykiewicz *et al.* (1963) suggested that caroverine may act directly on the smooth muscle, the mode of action of caroverine is still obscure. Therefore, experiments were undertaken to define the mode of the vasodilator action of caroverine fumarate on the basis of cellular calcium metabolism.

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Methods

Rat hindquarter preparation

Vascular reactivity of rats was investigated in hindquarter preparations perfused with the Krebs-Ringer solution containing (mM): NaCl 120.3, KCl 4.8, $MgSO_4$ 1.3, $CaCl_2$ 1.2, $NaHCO_3$ 25.2, KH_2PO_4 1.2, and dextrose 5.8; 4% dextran was also added in order to reduce the oedema in the perfused hindquarters. The medium was constantly aerated with 95% O_2, 5% CO_2 and maintained at 37°C (pH 7.4).

Rats were first anaesthetized with pentobarbitone (30 to 60 mg/kg, i.p.) and injected subcutaneously with heparin (5 mg/kg). Following a midline incision, the abdominal aorta and the inferior vena cava were isolated and prepared for cannulation. The abdominal aorta was rapidly cannulated with a polyethylene tube and connected to a perfusion system. Immediately after beginning perfusion of the hindquarter with the solution using a T-8 Sigmamotor pump, the

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inferior vena cava was cut open and a polyethylene tube was inserted to ensure a free outflow of the perfusate. Flow was adjusted to increase the perfusion pressure to 20 to 40 mmHg, which corresponded to flow values of 2 to 4 ml/min. The perfusion pressure was recorded on a Grass polygraph via Statham P-23 AA pressure transducer.

In the present experiments, the lumbar sympathetic chain was cut.

Aortic strips

The thoracic aorta was isolated from Wistar rats (either sex, weighing 200 to 300 g). The aorta was thoroughly cleaned of connective tissue without affecting the adventitial layer, and spiral strips were cut (2 to 2.5 mm in width and about 2.5 cm in length). The strips were suspended in a tissue bath containing 20 ml of Krebs Ringer solution maintained at 37°C and bubbled with a gas mixture of 95% O_2 and 5% CO_2. The change of isometric tension was recorded by means of a Grass (FT. 03) force-displacement transducer, connected to a Grass polygraph. The strips were subjected to a 1 g initial tension and this tension was maintained throughout the experiment. The experiments began after equilibration of the tissues for about 2 h.

Relaxation was studied on aortic strips maximally contracted by agonists. Relaxing agents were added to the solution in cumulative doses. Cumulative dose-response curves were obtained by stepwise increases in concentration of agents: the addition of agent was made as soon as a steady response was obtained to the preceding dose. The maximum contraction generated by agonists served as the control (100% contraction).

To determine the external calcium concentration-response curve, tissues were exposed to a calcium-free solution ($CaCl_2$ omitted from the solution for 60 min) and then $CaCl_2$ was added cumulatively. High-K (4×10^{-2} M) was added to the solution 15 min before addition of $CaCl_2$.

Cellular calcium uptake

Cellular calcium uptake of rat aorta was determined by a modified lanthanum method using ^{45}Ca (Karaki & Weiss, 1979). Rectangular strips about 10 mg each were made by cutting the aortic vessel longitudinally and were placed overnight in cold Krebs Ringer solution at 5°C. Before the experiment, strips were equilibrated for 4 h in the solution. After an incubation with various test solutions containing ^{45}Ca 1.25 μCi/ml, the strip was exposed to a 0.5°C lanthanum solution containing $LaCl_3$ 67.4 mM and buffered with Tris-HCl, pH 7.4, for 1 h. The strip was then digested by overnight incubation at 45 to 55°C with tissue solubilizer (Soluene-350, Packard). The solubilized sample was mixed with scintillator (Aquasol-2, New England Nuclear) and the radioactivity was determined by means of a liquid scintillation spectrometer (Tri-Carb 2003, Packard). ^{45}Ca was supplied by New England Nuclear Corporation.

Statistical analysis

Values were expressed or plotted as the mean ± s.e. unless otherwise stated and the statistical analysis was performed by Student's *t* test.

Drugs

Caroverine fumarate, 1-(2-diethylaminoethyl)-3-(*p*-methoxybenzyl)-1,2-dihydro-2-quinoxalinone fumarate, was supplied by Mitsubishi Chemical Industries, Ltd. (−)-Verapamil was kindly given by Eisai, Ltd. (−)-Noradrenaline bitartrate was purchased from Sigma.

Results

Rat hindquarter preparation

Figure 1 shows the inhibitory effect of caroverine fumarate (10^{-6} M) on the increased perfusion pressure brought about by treatment with potassium (8×10^{-2} M) or noradrenaline (10^{-6} M). The presence of caroverine (10^{-6} M) decreased both pressor responses to potassium and to noradrenaline. The inhibition of the response to potassium was much greater than that to noradrenaline, the response to potassium being reduced by 76.0 ± 2.6% ($n = 3$) while that to noradrenaline was reduced by 30.1 ± 2.4% ($n = 3$) by caroverine. Caroverine 10^{-6} M did not modify the basal perfusion pressure.

Rat isolated aorta

Figure 2 shows the inhibitory effect of caroverine on contractile responses to potassium (4×10^{-2} M) (high-K) and to noradrenaline (10^{-6} M) in rat isolated aortic strips. In the presence of caroverine (10^{-6} M) the contractile response to high-K, was greatly decreased, whereas the same treatment had little effect on the response to noradrenaline. Caroverine reduced high-K-induced contraction by 70.0 ± 6.4% ($n = 4$) and the noradrenaline-induced contraction by 7.0 ± 0.02% ($n = 6$).

Application of caroverine also caused relaxation in rat aortic strips already contracted with high-K (4×10^{-2} M) or with noradrenaline (10^{-6} M) in a dose-dependent manner (Figure 3). The ID_{50}s (50% inhibition of the maximum contraction induced by

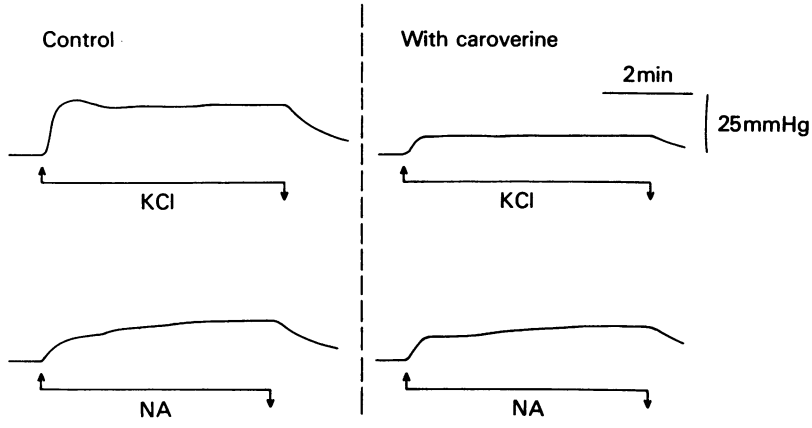

Figure 1 Effect of caroverine on the pressor responses induced by KCl (8×10^{-2} M) and noradrenaline (10^{-6} M) (NA) in the perfused rat hindquarter preparation. Caroverine (10^{-6} M) was introduced 10 min before application of KCl or NA. The curve represents the mean pressure.

high-K or noradrenaline) of caroverine were 2.4×10^{-7} M for the high-K response and 3.5×10^{-6} M for the noradrenaline response. Thus the inhibitory effect of caroverine was also greater on the strip contracted with high-K than on the strip contracted with noradrenaline.

Verapamil has been described as a selective inhibitor of transmembrane calcium influx (Kohldhardt, Bauer, Krause & Fleckenstein, 1972) and potassium-induced contraction in rabbit and rat aorta (Peiper, Griebel & Wende, 1971; Massingham, 1973; Golenhofen & Hermstein, 1975; Karaki, Kubota & Urakawa, 1979). Therefore, the effect of caroverine on the mechanical response was compared with that of verapamil.

Verapamil was applied cumulatively after high-K had caused a maximum contraction. Verapamil also caused relaxation in a dose-dependent manner (Figure 3). The ID_{50} for verapamil was 6.6×10^{-8} M. On the basis of the ID_{50}, the potency of caroverine was approximately half that of verapamil.

Concentration-response curve for external calcium

The cumulative application of calcium caused contraction of rat aortic strips after prior incubation in calcium-free solution with high-K. Figure 4 shows parallel shifts to the right of the concentration-response curve for calcium caused by caroverine 3×10^{-7} and 10^{-6} M, indicating competitive inhi-

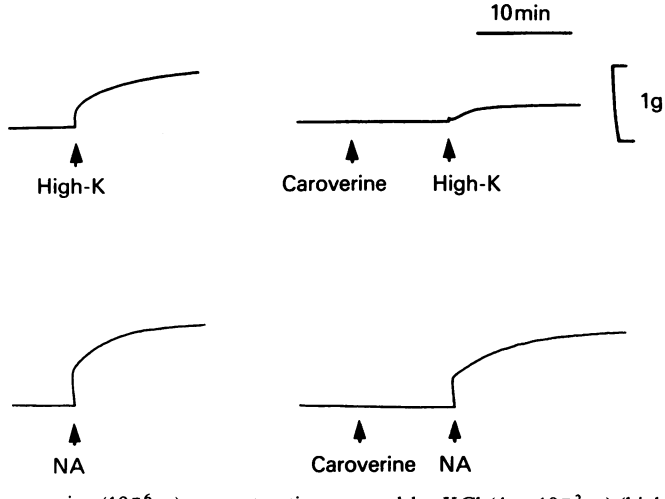

Figure 2 Effect of caroverine (10^{-6} M) on contractions caused by KCl (4×10^{-2} M) (high-K) and noradrenaline (10^{-6} M) (NA) in the rat isolated aorta. Caroverine was applied 10 min before treatment with high-K or NA.

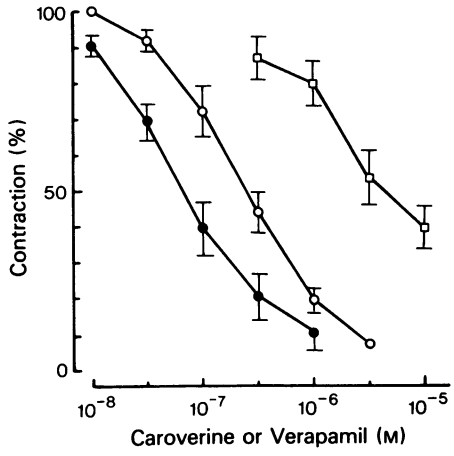

Figure 3 The concentration-inhibition relationships for caroverine or verapamil in the rat aorta: (○) and (□) represent effects of caroverine on high-K- and noradrenaline (NA)-induced contractions respectively; (●) the effect of verapamil on the high-K-induced contraction. Caroverine or verapamil was applied cumulatively after the contractile response had reached maximum. Maximum contractions caused by high-K (4×10^{-2} M) and NA (10^{-6} M) represent 100% contraction. Each point represents the mean of 4 experiments; vertical lines indicate s.e. mean.

bition. The negative logarithm of the affinity (pA_2) of caroverine was 7.01 ± 0.08 ($n = 8$) according to the calculation described by Van Rossum (1963).

Cellular ^{45}Ca uptake

The effect of caroverine was compared with verapamil on ^{45}Ca uptake of rat aorta. Figure 5 shows that high-K increased ^{45}Ca uptake from 345 ± 18 to 478 ± 32 μmol/kg wet wt. ($n = 8$). This increase was significant ($P < 0.01$). Caroverine (3×10^{-6} M) and verapamil (10^{-6} M) significantly ($P < 0.01$) inhibited the increase in the ^{45}Ca uptake induced by high-K, ^{45}Ca uptake being 410 ± 13 and 409 ± 15 μmol/kg wet wt. ($n = 8$), respectively. Caroverine (3×10^{-6} M) and verapamil (10^{-6} M) had no significant effect on the amount of ^{45}Ca uptake of control tissues which were not exposed to high-K.

Washout of caroverine and verapamil

Following maximum inhibition of high-K induced contraction by caroverine (3×10^{-6} M) or verapamil (10^{-6} M) treatment, both agents were washed out from the tissue with high-K solution which contained no drug. After the washout the depressed contractile response to high-K was progressively restored (Figure 6). At 1 h after the washout of caroverine, the

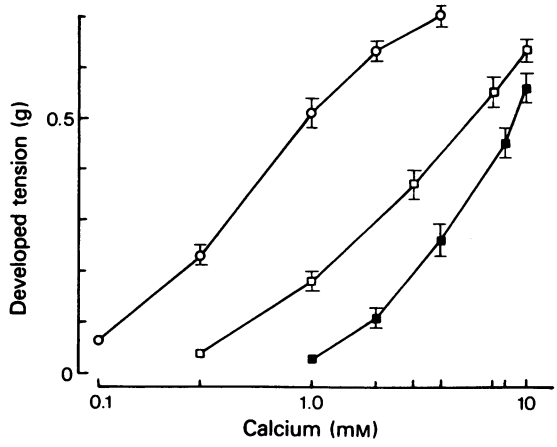

Figure 4 Effect of caroverine on the concentration-response curve for external calcium in the rat aorta treated with high-K. The muscle was treated with Ca-free solution for 60 min before the cumulative application of calcium. Caroverine or high-K was introduced 15 min before application of calcium. (○) Control; (□) and (■) treatment with caroverine, 3×10^{-7} and 10^{-6} M respectively. Control points and those in the presence of caroverine represent means of 8 and 4 experiments respectively; vertical lines indicate s.e. mean.

high-K-induced contraction was almost completely restored, while the verapamil-induced relaxation was only partially restored (Figure 6). Also, the rate of

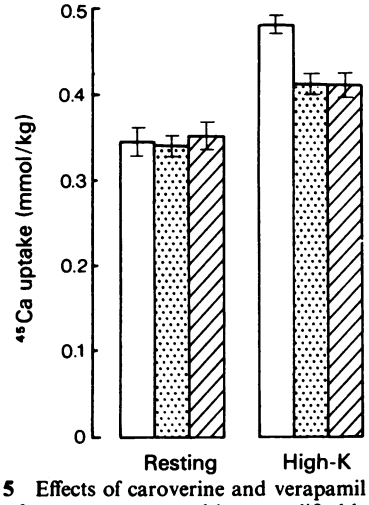

Figure 5 Effects of caroverine and verapamil on ^{45}Ca uptake of rat aorta measured by a modified lanthanum method. The uptake of the aorta was measured during 30 min incubation in the radioactive solution. Caroverine (3×10^{-6} M) (stippled column) or verapamil (10^{-6} M) (hatched column) was applied 10 min before soaking the muscles in the radioactive solution; open column: control.

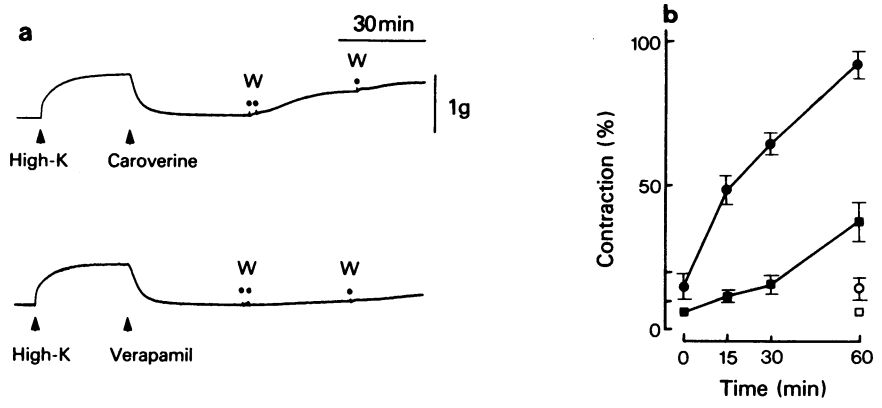

Figure 6 Effect of washout of caroverine or verapamil from rat aortic muscles treated with high-K: (a) shows typical result when muscles were treated with caroverine (3×10^{-6} M) or verapamil (10^{-6} M); (b) is the statistical presentation of results: (●) and (■) represent responses after the washout of caroverine (3×10^{-6} M) and of verapamil (10^{-6} M) respectively. At 0 and 30 min in (b), both agents were washed out twice and once respectively, as designated by W in (a). Open symbols represent responses without the washout of caroverine (○) and verapamil (□). Caroverine and verapamil were applied for 40 min before the first washout. Vertical bars represent 2 × s.e. ($n = 3$).

restoration was much faster in the caroverine-treated strip than in the verapamil-treated one. The inhibitory effect of caroverine or verapamil lasted for at least 2 h when the agent was present in the solution.

Discussion

In the present experiments, it was shown that caroverine fumarate caused vasorelaxation in hindquarter and isolated aortic preparations of rat. In addition, such inhibitory action of caroverine was much greater on high-K-induced contraction than on noradrenaline-induced contraction. Similar vascular relaxant actions of caroverine have been observed in the canine cerebral artery (Oh & Widauer, 1977).

Hornykiewicz *et al.* (1963) previously found that caroverine, like papaverine, has a spasmolytic action due to a direct effect on the smooth muscle. However, it was reported that the potency of the relaxant effect of papaverine was not different between high-K- and noradrenaline-induced contractions of rat aorta (Peiper *et al.*, 1971). However, in the present experiments caroverine caused a much greater relaxation of the high-K-induced contraction and had little effect on the noradrenaline-induced contraction at concentrations less than 10^{-6} M. This leads us to speculate that the mode of relaxant action of caroverine is different from that of papaverine.

It was proposed that in vascular smooth muscle, high-K-induced contraction is due to an increment of calcium influx caused by membrane depolarization while noradrenaline-induced contraction is attributed to the facilitation of release of the cellularly sequestered calcium (Van Breemen, Farinas, Gerba & MacNaughton, 1972). On the basis of this assumption, it seems unlikely that the mechanisms of the vasocontraction induced by both agonists (high-K and noradrenaline) are similar. The organic calcium influx inhibitor, verapamil, also had a greater inhibitory effect on high-K-induced contraction in vascular smooth muscles (Peiper *et al.*, 1971; Massingham, 1973; Golenhofen & Hermstein, 1975; Karaki *et al.*, 1979). These results suggest that caroverine, unlike papaverine, selectively inhibits calcium influx and such an inhibitory action plays an important role in the relaxant effect of caroverine.

Furthermore, caroverine attenuated the contractile response to external calcium in a competitive manner. In addition, the radioisotope study indicates that caroverine inhibited the increased ^{45}Ca uptake in high-K-treated tissues to the same level as that observed in verapamil-treated tissues. These results provide further evidence in support of the hypothesis that caroverine specifically inhibits calcium influx in vascular smooth muscles.

Compared with verapamil, the potency of the relaxant action of caroverine was half that of verapamil. Also, the tissue treated with caroverine recovered more rapidly from relaxation than the verapamil-treated tissue following a washout of the agent even when employed at a three times higher concentration. This property of caroverine may be advantageous for its clinical and experimental uses as a calcium antagonist.

In conclusion, the vascular relaxation induced by caroverine, like verapamil, results from the inhibition of the transmembrane calcium influx of smooth muscle.

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