Vascular Dilatory Action of the Chinese Crude Drug. II. Effects of Scoparone on Calcium Mobilization

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Further experiments were conducted to examine the effect of scoparone (6,7-dimethoxycoumarin), a coumarin derivative found in the Chinese crude drug "Capillaris Flos," on calcium mobilization. Scoparone does not affect Ca^{2+} influx through the voltage-dependent channel due to membrane depolarization. Its inhibitory action may be dependent not only on the inhibition of Ca^{2+} release from sarcoplasmic reticulum but also on the inhibition of extracellular Ca^{2+} influx through the receptor-operated channel.

Introduction

Scoparone, a coumarin isolated from Capillaris Flos (*Artemisia capillaris*), has been reported[1] to inhibit the contraction of vascular smooth muscle induced by norepinephrine (NE), 5-hydroxytryptamine (5-HT), histamine and angiotensin-II. Scoparone also produces a significant fall of blood pressure,[2] but the mechanism remains obscure. To clarify the mode of action of scoparone, further experiments were conducted to examine its effect on calcium mobilization in vascular smooth muscle.

Experimental

Male white rabbits weighing about 2.5 kg were bled to death by severing both carotid arteries, and the thoracic aorta (TA) and ear artery (EA) were removed and cut into helical strips (3—5 mm × 15—20 mm). Rat TA and portal vein (PV) were isolated from male Wistar rats weighing about 250 g. Rat TA was cut into helical strips (2 mm × 15—20 mm) and PV was cut into tubular strips (20 mm).

Physiological Salt Solution Contained (mM): NaCl 120.1, KCl 4.8, $MgSO_4$ 1.3, $CaCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.2 and glucose 5.8. It was aerated with a 95% O_2–5% CO_2 gas mixture and kept at 37 °C and pH 7.4. Calcium-free solution was prepared by removing $CaCl_2$ with 0.1 mM ethylene glycol bis(β-aminoethylether)-$N,N;N',N'$-tetracetic acid (EGTA).

To investigate the mechanical response, each preparation was suspended in an organ bath (25 ml) and subjected to an initial load of about 2 g. A 1.5-h equilibration period was allowed before initiation of the experiments. During this period, the solutions were replaced every 30 min. Contractions were recorded isometrically *via* a force-displacement transducer (Nihon Denki Sanei, Tokyo, Japan).

Drugs used were norepinephrine hydrochloride (Sigma), clonidine hydrochloride (Sigma), nifedipine (Sigma), nitroglycerin (Nihon Kayaku), EGTA (Dojindo Laboratories) and $^{45}CaCl_2$ (New England Nuclear). All other drugs were obtained from Wako Pure Chemical Industries. Scoparone was extracted and purified by our method.[3] Scoparone was dissolved in acetone; the final concentration of acetone in the bath did not exceed 0.1% and had no effect on muscle contraction.

Statistical analysis was performed by Dunnett's method.[4] Results are expressed or plotted as the mean ± S.E.

1) Effects on the NE-Induced Contraction (Rabbit TA and EA or Rat TA and PV) The maximum contractions induced by NE at 10^{-6} (TA and EA) or 10^{-5} M (PV) in the preparation in a bathing medium containing 10^{-6} M propranolol were taken as 100% contractile force. The tissues were then washed 3 times with physiological salt solution at 15 min intervals and pretreated with each test drug for 10 min, then NE (rabbit or rat TA and rabbit EA, 10^{-9}—3×10^{-5} M; rat PV, 10^{-7}—10^{-4} M) was cumulatively added to the bathing medium containing 10^{-6} M propranolol.

2) Effects on the NE-Induced Phasic Contraction (Rabbit TA and EA) Tissues were incubated in a Ca^{2+}-free medium with 0.1 mM EGTA for 30 min and then NE at 10^{-6} M was added to the bathing medium containing 10^{-6} M propranolol. Each test drug was added to the bath 10 min before the application of NE. The contraction induced by NE

(10^{-6} M) in a normal Ringer medium containing 10^{-6} M propranolol was taken as 100% contractile force.

3) Effects on the NE-Induced Tonic Contraction (Rabbit TA and EA) Tissues were incubated in a Ca^{2+}-free medium with 0.1 mM EGTA for 30 min and then NE (10^{-6} M) was added to the bathing medium containing 10^{-6} M propranolol and 10^{-6} M nifedipine. Each test drug was added to the bath 10 min before the application of NE. $CaCl_2$ (0.1— 1.2 mM) was added in a cumulative manner to induce nifedipine-resistant NE-activated contraction.

4) Effects on the Clonidine-Induced Contraction (Rabbit TA and EA) The maximum contractions induced by clonidine at 10^{-6} M in the preparation in the bathing medium containing 10^{-6} M propranolol are taken as 100% contractile force. The tissues were then washed 3 times with physiological salt solution at 15 min intervals and pretreated with test drugs for 10 min, then clonidine (10^{-8}—3×10^{-5} M) was cumulatively added to the bathing medium containing 10^{-6} M propranolol.

5) Effects on the KCl-Induced Contraction (Rabbit TA and EA) The maximum contractions induced by KCl at 60 mM in the preparation are taken as 100% contractile force. The tissues were then washed 3 times with physiological salt solution at 15 min intervals and pretreated with test drugs for 10 min, then KCl (10—60 mM) was cumulatively added to the bathing medium.

6) Effect on the Contractile Responses to $CaCl_2$ in Depolarized Muscles (Rabbit TA and EA) Tissues were incubated in a Ca^{2+}-free (0.1 mM ethylenediaminetetraacetic acid (EDTA)) medium for 60 min and then 50 mM KCl was added to the bathing medium. Thereafter, $CaCl_2$ (0.1— 1.2 mM) was cumulatively added to the bath. The maximum contraction induced by $CaCl_2$ at 1.2 mM was taken as 100% contractile force.

7) Effects on $^{45}Ca^{2+}$ Uptake (Rabbit TA) The method used in this experiment was similar to the "low-temperature lanthanum method" described by Karaki *et al.*[5] Specifically, rabbit TA (3—5 × 8—10 mm) was first incubated for 60 min in physiological solution containing 1.2 mM $^{45}CaCl_2$ (0.5—1.0 μCi/ml), 50 ml KCl and a test drug and then rinsed for 60 min in ice cold lanthanum solution (LaCl₃ 67.4 mM, glucose 11.1 mM, Tris–HCl 12.5 mM, pH 7.4, aerated with 100% O_2). Each tissue was then placed in a scintillation vial containing 0.5 ml of tissue solubilizer (Soluene 350) for 24 h at room temperature, and after the addition of the scintillation mixture, radioactivity was counted in a liquid scintillation counter (LSC-903, Aloka).

Results

Scoparone (10^{-5}—10^{-4} M) inhibited the responses to NE in the presence of 10^{-6} M propranolol in a concentration-dependent manner and shifted the concentration-response curve to the right (Fig. 1).

The pA_2 values of scoparone in rabbit TA and EA were 5.60 and 5.73, respectively. Further, the pA_2 values of nitroglycerin (GTN) in rabbit TA and EA were 7.45 and 7.06, respectively.

In rat TA, in the presence of 10^{-6} M propranolol, scoparone (10^{-5}—10^{-4} M) and GTN (10^{-7}, 10^{-6} M) signif-
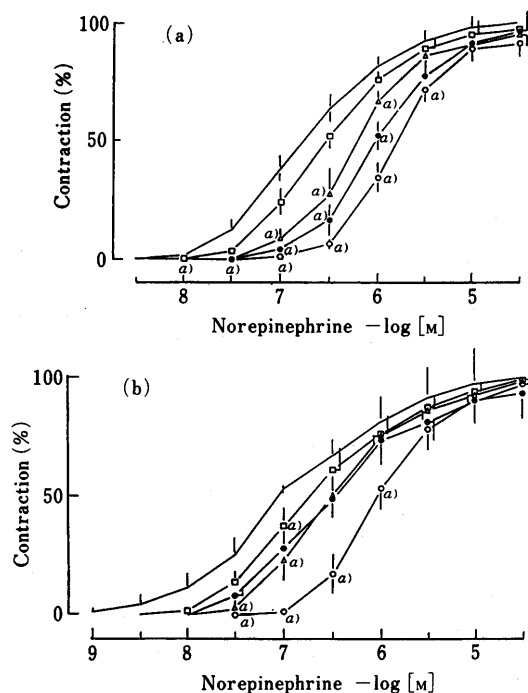
Fig. 1. Effects of Scoparone on the Contractile Response of Norepinephrine under 10^{-6} M Propranolol in the Isolated Rabbit Thoracic Aorta (a) and Ear Artery (b) ($n=6$, $p^* < 0.01$)

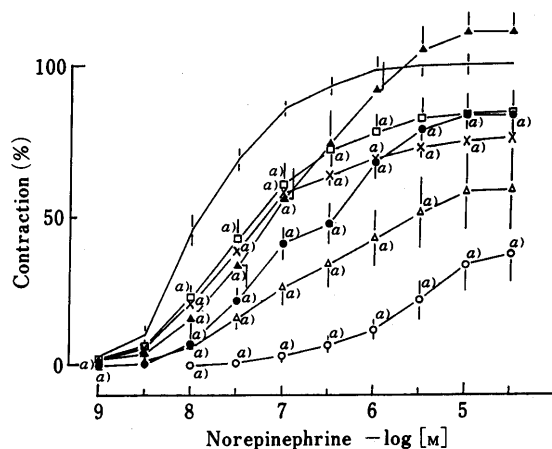———, control; —□—, 10^{-5} M scoparone; —△—, 3×10^{-5} M scoparone; —○—, 10^{-4} M scoparone; —●—, 10^{-6} M nitroglycerin.

Fig. 2. Effects of Scoparone, Nitroglycerin and Nifedipine on the Contractile Response of Norepinephrine under 10^{-6} M Propranolol in the Isolated Rat Thoracic Aorta ($n=6$—8, $p^* < 0.01$)

———, control; —○—, 10^{-4} M scoparone; —△—, 3×10^{-5} M scoparone; —□—, 10^{-5} M scoparone; —●—, 10^{-6} M nitroglycerin; —▲—, 10^{-7} M nitroglycerin; —×—, 10^{-7} M nifedipine.
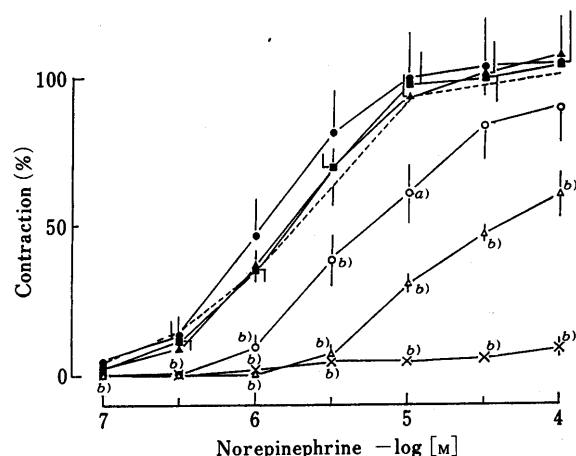

Fig. 3. Effects of Scoparone, Nitroglycerin and Nifedipine on the Contractile Response of Norepinephrine under 10^{-6} M Propranolol in the Isolated Rat Portal Vein ($n=5$—7, $p^* < 0.05$, $p^{**} < 0.01$)

------, control; —○—, 10^{-4} M scoparanone; —△—, 3×10^{-4} M scoparone; —●—, 10^{-6} M nitroglycerin; —▲—, 10^{-5} M nitroglycerin; —■—, 10^{-4} M nitroglycerin; —×—, 10^{-7} M nifedipine.
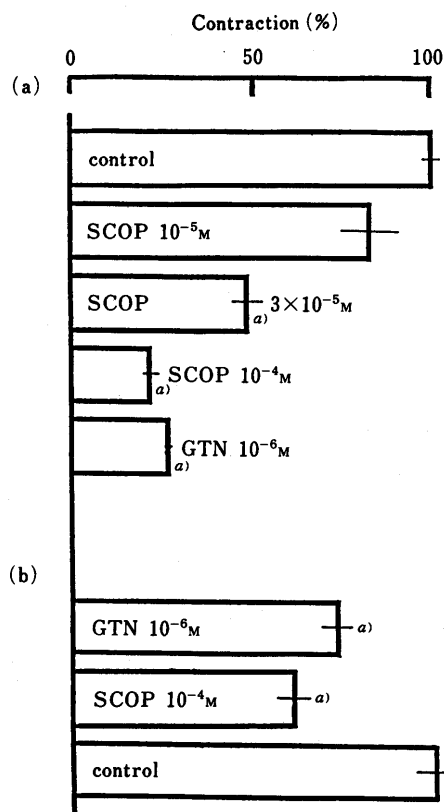

Fig. 4. Effects of Scoparone on the Phasic Contraction of 10^{-6} M Norepinephrine in the Isolated Rabbit Thoracic Aorta (a) and Ear Artery (b) Exposed to Ca^{2+}-Free Medium with 0.1 mM EGTA ($n=6$—8, $p^* < 0.01$)

SCOP, scoparone; GTN, nitroglycerin.

icantly inhibited the responses to NE in a concentration-dependent manner. Nifedipine at 10^{-7} M also inhibited the NE responses. The pD$'_2$ values were 4.28 for scoparone, 5.30 for GTN and 6.52 for nifedipine (Fig. 2). In PV, scoparone (1×10^{-4}, 3×10^{-4} M), like nifedipine at 10^{-7} M, inhibited the responses to NE in a concentration-dependent manner, with a pD$'_2$ value of 3.22, but GTN at 10^{-4} M had no effect (Fig. 3).

Scoparone (10^{-5}—10^{-4} M) significantly inhibited the phasic response to NE in the presence of 10^{-6} M propranolol, in a concentration-dependent manner (Fig. 4).

In rabbit TA, the phasic response to NE was inhibited by scoparone at 10^{-5}, 3×10^{-5} and 10^{-4} M to $82.4 \pm 8.3\%$, $47.9 \pm 4.2\%$ and $21.9 \pm 1.4\%$ of the control and by GTN at 10^{-6} M to $26.8 \pm 0.9\%$.

In EA, scoparone at 10^{-4} M and GTN at 10^{-6} M inhibited the response to $59.9 \pm 4.5\%$ and $71.8 \pm 4.2\%$, respectively, of the control.
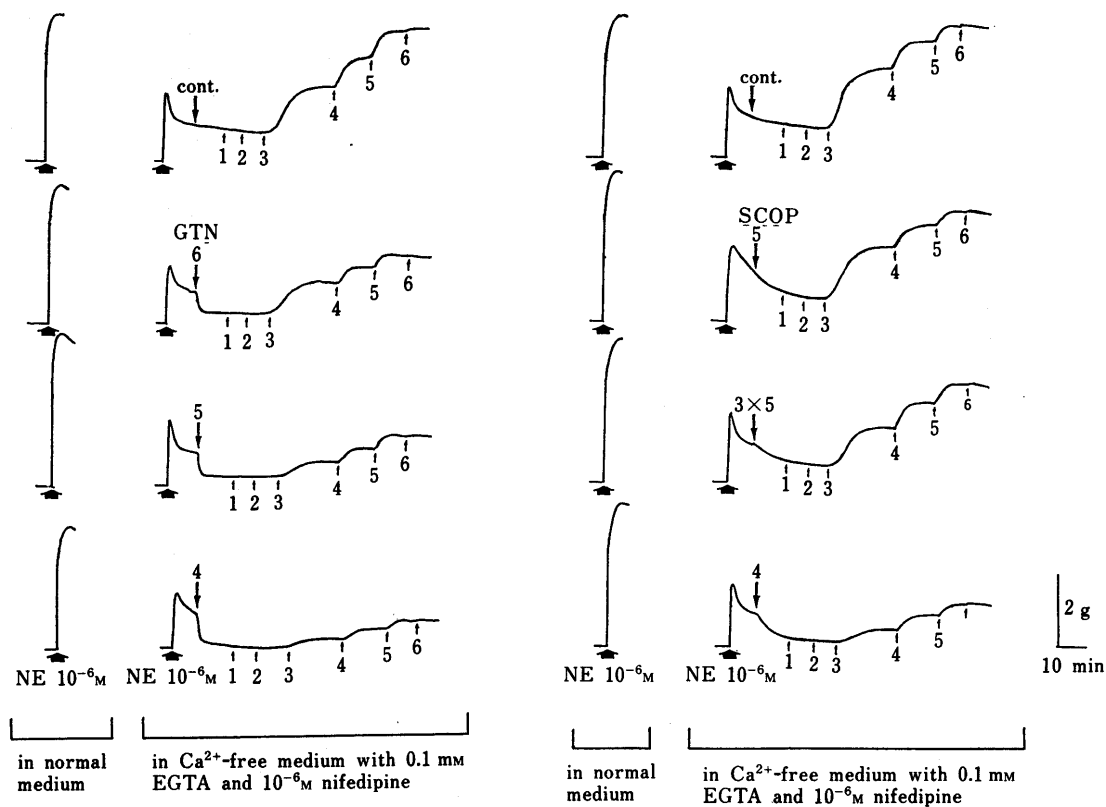
Fig. 5.   Effects of Scoparone at 10^{-4} M on the Tonic Contraction (Nifedipine-Resistant Contraction) in the Isolated Rabbit Thoracic Aorta

1, Ca^{2+} 0.1 mM; 2, 0.2 mM; 3, 0.3 mM; 4, 0.5 mM; 5, 1.0 mM; 6, 1.2 mM. NE, norepinephrine; GTN, nitroglycerin; SCOP, scoparone. 6, 10^{-6} M; 5, 10^{-5} M; 3×5, 3 × 10^{-5} M; 4, 10^{-4} M.
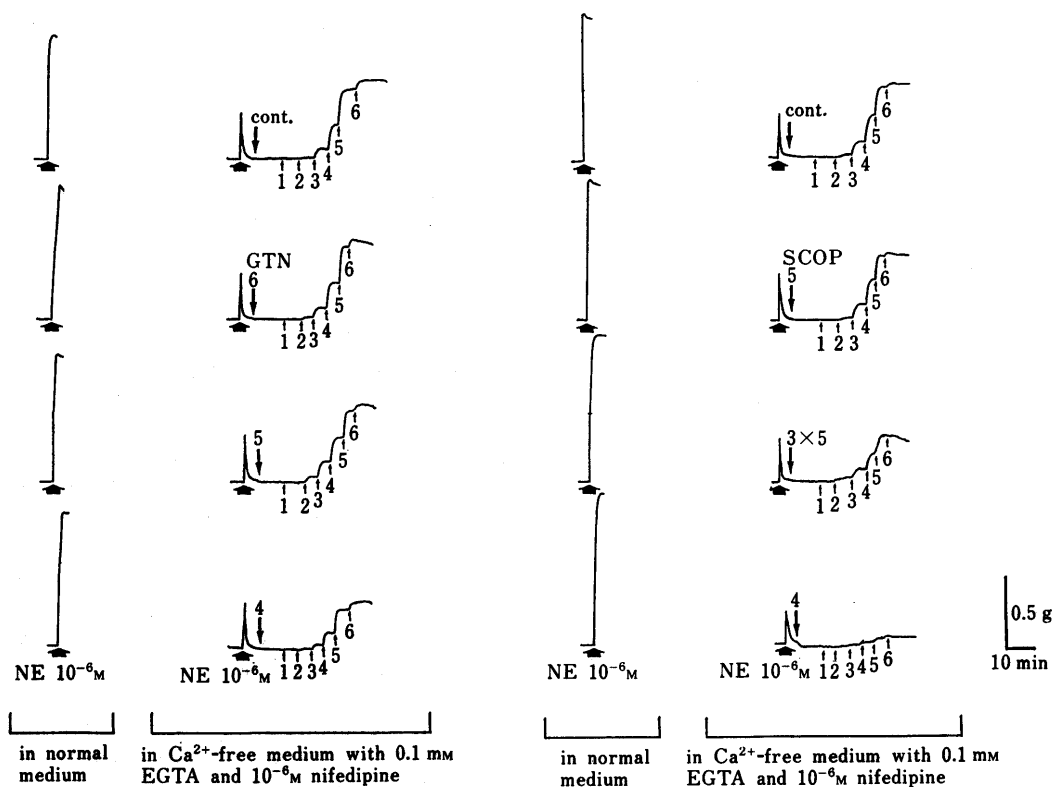

Fig. 6.   Effects of Scoparone at 10^{-4} M on the Contraction (Nifedipine-Resistant Contraction) in the Isolated Rabbit Ear Artery

1, Ca^{2+} 0.1 mM; 2, 0.2 mM; 3, 0.3 mM; 4, 0.5 mM; 5, 1.0 mM; 6, 1.2 mM. NE, norepinephrine; GTN, nitroglycerin; SCOP, scoparone. 6, 10^{-6} M; 5, 10^{-5} M; 3×5, 3 × 10^{-5} M; 4, 10^{-4} M.
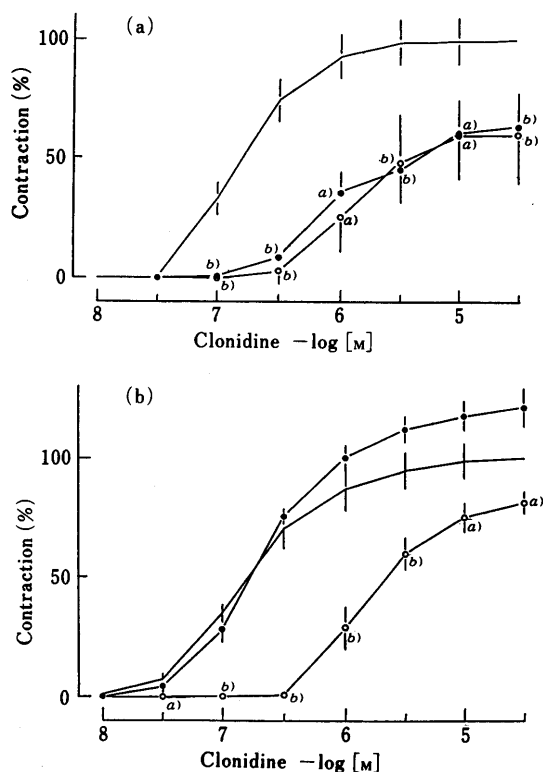
Fig. 7. Effects of Scoparone and Nitroglycerin on the Contractile Response of Clonidine under 10^{-6} M Propranolol in the Isolated Rabbit Thoracic Aorta (a) and Ear Artery (b) ($n = 6$, $p^* < 0.05$, $p^{**} < 0.01$)

———, control; —O—, 10^{-4} M scoparone; —●—, 10^{-6} M nitroglycerin.

Fig. 9. Effects of Scoparone and Nitroglycerin on the Contractile Response of $CaCl_2$ in the Rabbit Thoracic Aorta (a) and Ear Artery (b) Depolarized by 50 mM KCl ($n = 5$—7, $p^* < 0.05$, $p^{**} < 0.01$)

———, control; —O—, 10^{-4} M scoparone; —●—, 10^{-6} M nitroglycerin.

Fig. 10. Effects of Scoparone (SCOP), Nitroglycerin (GTN) on $^{45}Ca^{2+}$ Uptake of Rabbit Thoracic Aorta in Normal and High K^+ Medium
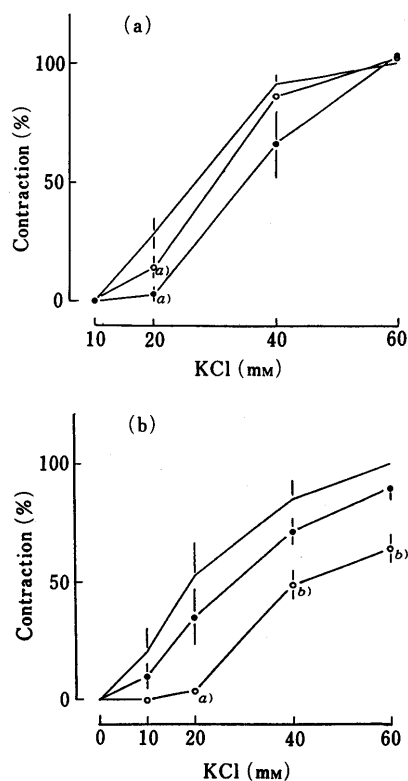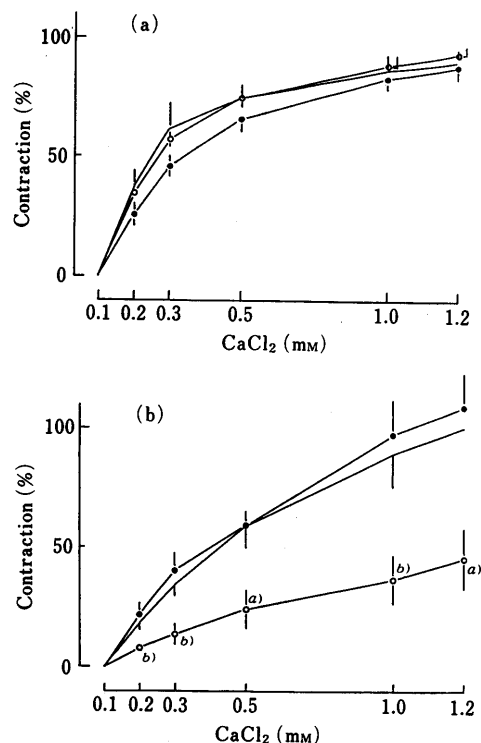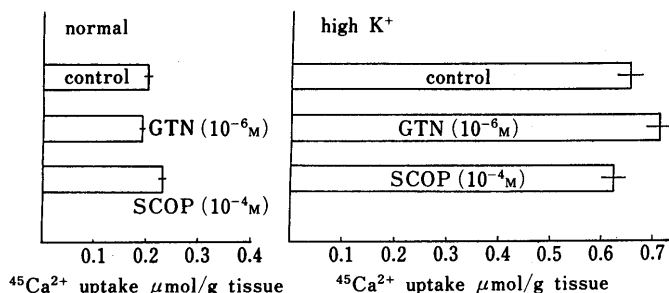

Fig. 8. Effects of Scoparone and Nitroglycerin on the Contractile Response of KCl in the Rabbit Thoracic Aorta (a) and Ear Artery (b) ($n = 6$—8, $p^* < 0.01$)

———, control; —O—, 10^{-4} M scoparone; —●—, 10^{-6} M nitroglycerin.

In rabbit TA and EA, scoparone (10^{-5}—10^{-4} M) significantly inhibited the 10^{-6} M NE-activated, nifedipine-resistant tonic Ca^{2+} response, in a concentration-dependent manner.

In TA, GTN at 10^{-6}—10^{-4} M inhibited the tonic response in a concentration-dependent manner, as in the case of scoparone, but GTN at 10^{-4} M in EA moderately inhibited the tonic response (Figs. 5, 6).

In rabbit TA and EA, scoparone at 10^{-4} M significantly inhibited the responses to clonidine in the presence of 10^{-6} M propranolol, with pD'_2 values of 3.83 in TA and 3.36 in EA. Similarly, GTN at 10^{-6} M significantly inhibited the response to clonidine in TA with a pD'_2 value of 5.77, but it did not inhibit the response in EA (Fig. 7).

In TA, scoparone at 10^{-4} M only inhibited the response to a low concentration of KCl (20 mM), but like GTN at 10^{-6} M, scoparone had almost no effect on the KCl- and $CaCl_2$-induced contractions. In EA, however, scoparone at 10^{-4} M significantly inhibited the responses to KCl and to $CaCl_2$. GTN at 10^{-6} M did not have any effect, as in TA

(Figs. 8 and 9).

Scoparone at 10^{-4} M and GTN at 10^{-6} M did not have any significant effect on $^{45}Ca^{2+}$ uptake in the normal medium or in high-K^+ medium (Fig. 10).

Discussion

Contractile responses induced by NE in vascular smooth muscles are thought to be due to binding of NE to α_1-receptors on vascular smooth muscles, leading to breakdown of phosphoinositides[6] into inositol-1,4,5-triphosphates and 1,2-diacylglycerol as second messengers to cause Ca^{2+} release from sarcoplasmic reticulum (SR) and Ca^{2+} influx through the receptor-operated channel (ROC), respectively.[7-9] Further, a phasic response induced by NE in Ca^{2+}-free medium is due to Ca^{2+} release from SR activated by α_1-adrenoceptors.[10-12] Scoparone at 10^{-5} M and higher concentrations inhibited this phasic response to NE in a concentration-dependent manner, suggesting an inhibitory effect on Ca^{2+} release from SR activated by α_1-receptor. In addition, the effect of scoparone was examined on Ca^{2+} influx through ROC based on the effect on NE-activated, nifedipine-insensitive tonic Ca^{2+} responses. In TA and EA, scoparone at 10^{-5}—10^{-4} M inhibited the Ca^{2+} response in a concentration-dependent manner, but GTN at 10^{-4} M in EA produced only a small inhibition. These results suggest that, as reported by Akimoto,[13] the tonic response due to activation of ROC by NE in rabbit EA is resistant to GTN and that scoparone inhibits the nifedipine- and GTN-resistant contraction. Further, the results indicate that there is difference in Ca^{2+} mobilization between TA and EA.

In order to examine the mechanism of action of scoparone and GTN, and to analyze the differences in Ca^{2+} mobilization in rabbit TA and EA, the effects on the clonidine responses in rabbit TA and EA and on the NE responses in rat TA and PV were examined. The results indicated that GTN at 10^{-6} M inhibited the clonidine response in rabbit TA but not in EA. In addition, GTN at 10^{-7} and 10^{-6} M significanlty inhibited the NE response in rat TA, but even at 10^{-4} M it had no effect on the NE response in PV. Scoparone (10^{-5}—3×10^{-4} M), however, inhibited these responses uniformly. Vascular responses to clonidine, a preferential α_2-adrenoceptor agonist, is due to activation of post junctional α_2-adrenoceptors resulting in extracellular Ca^{2+} influx.[14] Similarly, in rat PV, SR is less developed and its contractile response depends on extracellular Ca^{2+} influx.[15] These results suggest that GTN is insensitive to the responses due to extracellular Ca^{2+} influx and its inhibitory action depends mainly on the inhibition of Ca^{2+} release from SR. The inhibitory action of scoparone, however, may be dependent not only on the inhibition of Ca^{2+} release from SR but also on the inhibition of extracellular Ca^{2+} influx, indicating more flexibility than GTN. In general, vascular smooth muscles are thought to contain Ca^{2+} necessary for contraction to a large degree in intracellular Ca^{2+} stores, but according to Sutter et al.,[16] dependency on extracellular Ca^{2+} increases in small vascular arterial smooth muscles. Therefore, one of the differences in Ca^{2+} mobilization between rabbit TA and EA may be the dependency on Ca^{2+} stores in rabbit TA and dependency on extracellular Ca^{2+} influx for contractions in EA.

Rat PV, which is insensitive to GTN, is known not to be affected by guanylate cyclase activity as much as rat TA, which has high activity of guanylate cyclase.[17] In addition, GTN was recently reported to activate guanylate cyclase, leading to an increased level of cyclic guanosine monophosphate (cGMP) resulting in stimulation of efflux of intracellular Ca^{2+}.[18,19] These results raise the possibility that the differences in the sensitivity of GTN in rat TA and PV may be due to the difference in the activity of guanylate cyclase. In the present experiments, the results that rabbit EA and rat PV showed some resistance to GTN may suggest a low activity of guanylate cyclase in rabbit EA, as in rat PV.

Vasoconstriction induced by a high concentrations of KCl is known to be due to influx of extracellular Ca^{2+} through VDC.[20,21] In the present experiments, the effects of drugs were examined on the KCl response and the $CaCl_2$ response as vasocontractions related to VDC and on $^{45}Ca^{2+}$ uptake in rabbit TA, in which 2 types of calcium channels, VDC and ROC, have been pharmacologically clarified.[8] The results indicated that scoparone at 10^{-4} M and GTN at 10^{-6} M did not affect these responses, suggesting scoparone, like GTN, does not affect Ca^{2+} influx through VDC due to membrane depolarization as much as Ca^{2+} influx due to receptor activation. However, the action of scoparone at 10^{-4} M is different from that of GTN in that scoparone significantly inhibited the responses to KCl and $CaCl_2$ only in EA. This may be due to the fact that, in rabbit EA, the single channel conductance of VDC is about 15 pS, which is about 3 times the conductance of ROC,[22] possibly suggesting a higher sensitivity of EA of VDC than ROC. However, this point can not be clarified from the present experiments.

References and Notes

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