A Phenoxazine Compound, 2-Amino-4,4α-dihydro-4α-7-dimethyl-3*H*-phenoxazine-3-one Reverses the Phenylephrine or High-K^+ Induced Contraction of Smooth Muscles in Rat Aorta and Guinea Pig Tenia Cecum

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2-Amino-4,4α-dihydro-4α-7-dimethyl-3*H*-phenoxazine-3-one (Phx-1) reversed concentration dependently the contraction of both rat aorta and guinea pig tenia cecum induced by phenylephrine or high-K^+. Since Phx-1 suppresses the responses of human mononuclear cells to phytohemagglutinin, (Akazawa *et al. Tohoku J. Exp. Med*., 196, 185—192, 2002), Phx-1 may be useful for developing new vasorelaxing agents with immunosuppressive action.

Key words smooth muscle; aorta; tenia cecum; phenoxazine

2-Amino-4,4α-dihydro-4α-7-dimethyl-3*H*-phenoxazine-3-one (Phx-1) is a yellowish brown compound synthesized by the reaction of human or bovine hemoglobin with 2-amino-5-methylphenol,[1] and is relatively soluble in water, while chemically synthesized phenoxazines are generally insoluble to water. Phx-1 has been shown to exert various biological effects such as immunosuppressive effects,[2—4] anticancer effects,[5—7] and anti-viral activity.[8,9] These actions were shown to be exhibited more than several hours after the exposure of cells to Phx-1. However, the biological effect of Phx-1 during the short term has not been investigated.

On the other hand, Huang *et al*.[10,11] demonstrated that emodin (1,3,8-trihydroxy-6-methylanthraquinone) and scoparone (6,7-dimethoxycoumarin) isolated from *Polygonum multiflorum* and *Artemisia scoparia*, respectively, possess immunosuppressive activity and exert vasorelaxant effects on the rat aorta. Especially, the vasorelaxing effect of emodin occurred within the short period, and considered to be caused by hydrogen peroxide produced from emodin itself, because emodin conforms to the semiquinone. Though Phx-1 is not a semiquinone, its chemical structure is similar to emodin, having a methyl group at the same position of the tricyclic chromophore. Taking account of the immunosuppressive effects of Phx-1,[2—4] it is conceivable that Phx-1 may have vasorelaxing activity in the short term. It may be of significance to investigate the relationship between chemical structure and activities of Phx-1 and emodin. Present report firstly deals with the reversing activities of Phx-1 against the contraction of rat aorta and guinea pig tenia cecum induced by high K^+ or phenyephrine.

MATERIALS AND METHODS

All experimental procedures were performed in accordance with the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan. Results were presented as means±S.E.M.

Male rats (Wistar, 10—12 weeks) were sacrificed under deep anesthesia with diethyl ether. The thoracic aorta was isolated and the endothelial cells were removed by gently rubbing the aorta between fingers as described previously.[12] Helical strips (2—3 mm wide and 10 mm long) were prepared, and one end of the strip was connected to a tension transducer (BG-10, Kulite Semiconductor Products, Leonia, NJ, U.S.A.) to measure isometric tension. The strip was equilibrated in physiological salt solution (PSS) at 37 °C for 60 min. The PSS contained (mM) NaCl 119.8, KCl 5.4, $CaCl_2$ 2.5, $MgCl_2$ 1.0, NaH_2PO_4 1.3, $NaHCO_3$ 23.8 and glucose 5.6 and bubbled with 95% O_2: 5% CO_2 (pH 7.4). Muscle contraction was induced by addition of either 40 mM KCl or 1 μM phenylephrine to PSS for 30 min, and Phx-1 was applied cumulatively in the presence of KCl or phenylephrine. The maximum tension responses to high-K^+ or phenylephrine were regarded as 100% of tension.

Male Hartley guinea pig (200—500 g) were sacrificed under deep anesthesia with diethyl ether and the tenia ceci were removed. A small muscle layer strip (100—200 μm wide and 4.0—5.0 mm long) was attached to a pair of tungusten wires with silk thread monofilaments, one of which was connected to the tension transducer to measure isometric tension.[13] After the strip was equilibrated in PSS at 25 °C for at least 30 min, a high concentration of K^+ solution (125 mM K^+ prepared by equimolar substitution of NaCl by KCl) was applied to induce maximum tension, and then Phx-1 was applied cumulatively in the presence of high-K^+. The force levels were expressed as a percentage of maximum tension measured in the absence of Phx-1.

Phx-1 (MW: 242) was prepared as described previously,[1] and was dissolved in PSS from a 20 mg/ml stock solution in dimethyl-sulfoxide (DMSO). The chemical structure of Phx-1 is shown in Fig. 1, in comparison with that of emodin, 1,3,8-trihydroxy-6-methylanthraquinone. The highest final concentration of DMSO was 0.4%, which did not affect the tension measurements. Phenylephrine was purchased from Wako Pure Chemical Co. Ltd. (Tokyo). All other chemicals were of reagent grade.

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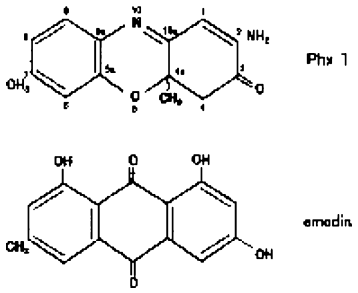

Fig. 1. Chemical Structure of Phx-1 and Emodin

RESULTS

Figure 2A shows the example tension traces measured from the aortic strips of the rat. Addition of either 40 mM KCl or 1 μM phenylephrine to PSS induced the increased tension that reached a plateau in approximately 30 min. Cumulative applications of Phx-1 caused relaxation of the strips in a concentration-dependent manner, irrespective of stimuli that triggered the contraction (cell membrane depolarization by high-K^+ or binding of phenylephrine to α-adrenoceptors). Since endothelial cells, which produces nitric oxide to cause vasorelaxation, are not present in the aortic strips of the rat, the relaxing effects of Phx-1 to aortic strips should be regarded as direct effects on the smooth muscles.

The tension of the rat aortic strips caused by either high-K^+ or phenylephrine was decreased according to the increase of Phx-1 concentrations (circles and solid dots in Fig. 2B). Phx-1 strongly inhibited the tension at 30 μg/ml (124 μM), and nearly complete inhibition was obtained at 80 μg/ml (331 μM). The EC_{50} value, defined as effective concentration to cause 50% inhibition, was estimated to be approximately 20 μg/ml (83 μM). These results clearly demonstrate that Phx-1 exerts vasorelaxant effects in the rat aorta.

Relaxing effects of various concentrations of Phx-1 for smooth muscle was also examined in guinea pig tenia cecum (Fig. 3). Phx-1 inhibited the tension in a concentration-dependent manner, though the concentration range of Phx-1 was limited in this experiment. At 20 μg/ml (83 μM), Phx-1 reduced the tension to, on the average, 61%, suggesting that the EC_{50} value was slightly higher than 20 μg/ml. Thus, the results in Figs. 2 and 3 support the views that Phx-1 causes relaxation of both vascular (rat aorta) and intestinal (tenia) smooth muscles at a similar concentration range.

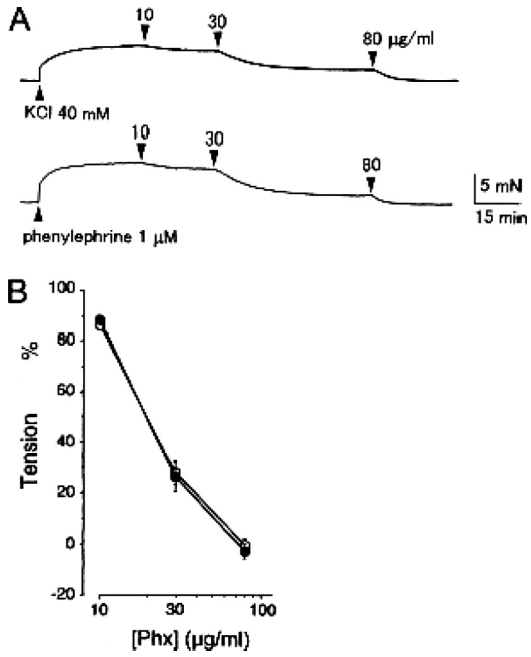

Fig. 2. Effects of Phx-1 on the Tension of Rat Aortic Strips

(A) Tension traces of rat aortic strips measured during high K^+ induced contraction (upper) and phenylephrine-induced contraction (lower). In each trace, application of 40 mM KCl or 1 μM phenylephrine at an upward arrowhead was followed by the addition of various concentrations of Phx-1 to the bath as indicated by downward arrowheads. (B) Concentration–response of the tension of rat aortic strips at various concentrations of Phx-1. The tension of rat aortic strips was measured at 37 °C. Each symbol represents mean±S.E.M. from 4 strips stimulated by KCl (circles) or phenylephrine (solid dots). Note that 10 μg/ml Phx-1 corresponds to 41 μM.

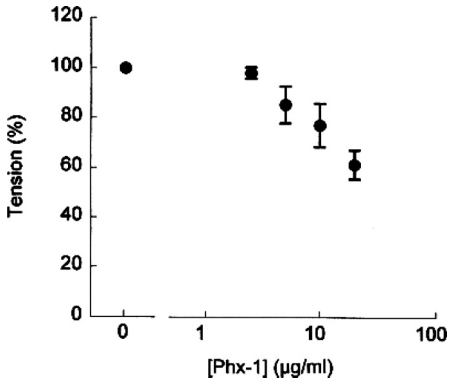

Fig. 3. Relation between Phx-1 Concentration and Tension Obtained from Guinea Pig Tenia Ceci (25 °C)

Each symbol represents mean±S.E.M. from 4 strips stimulated by KCl.

DISCUSSION

Phx-1 has been shown to inhibit the proliferation of phytohemagglutinin activated human peripheral blood mononuclear cells,[2] suppress the expression of cell surface IgM in DT40 B cell line[3] and inhibit the degranulation of mast cells.[4] These effects on the immunocytes of Phx-1 occur after at least 12 h, and may be characterized as the slow effects of the drug. In contrast, the present results indicate that Phx-1 showed its relaxing effects within 20—30 min (Fig. 2A), which may be characterized as fast effects. The results in Figs. 2 and 3 showed that Phx-1 acts as a relaxant of smooth muscles of rat aorta and guinea pig tenia cecum. Such activities of Phx-1 are similar to those of emodin, isolated from Chinesese herb. Namely, Huang *et al.*[10,11] reported that emodin, a tricyclic chromophore (the semiquinone with methyl group at C6; Fig. 1), exerts vasorelaxing and immunosuppressive activities. They suggested that the vasorelaxing effects of emodin might be caused by hydrogen peroxide produced from the semiquinone structure of emodin. However, Phx-1 is not the semiquinone, and does not produce hydrogen peroxide (our unpublished data). Thus, we consider that the methyl group at C7 of the phenoxazine ring, as emodin has, may contribute to the appearance of vasorelaxing activity of Phx-1, which should be further investigated.

Cyclosporine A is often used as an immunosuppressive agent and causes hypertension in organ transplant recipi-

ents.[14] This compound has been shown to induce vascular contraction and attenuate the relaxation response.[14] A drug to cause immunosuppression and exert beneficial effect on vascular tension, therefore, will be useful for the treatment of transplantation rejection. Although vasorelaxing effect of Phx-1 (EC_{50}: approximately 80 μM) seems somewhat weaker than that of emodin (EC_{50}: 14 μM),[11] Phx-1 may be useful as an immunosuppressant in clinical situations such as organ transplantation that require control of hypertension.

Inhibitory mechanisms of smooth muscle contraction by Phx-1 are not known at this point. Because the concentration–response relation of Phx-1 was very similar for contractions induced by high-K^+ and phenylephrine in the rat aorta (Fig. 3), it is conceivable that Phx-1 inhibits the processes that are commonly activated by depolarization and α-adrenoceptor stimulation. Potential sites of action, though speculation, include Ca^{2+} influx pathways, Ca^{2+} release from the intracellular stores, intracellular signal transduction pathways, phosphorylation/dephosphorylation of myosin light chain, and interaction between actin and myosin. Further studies are required to elucidate the mechanism of the drug action.

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