Comparison of the Relaxing Actions of Acetylcholine and Substance P in Smooth Muscle of the Guinea-Pig Aorta

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Abstract

The relationship between relaxation produced by acetylcholine (ACh) or substance P (SP) and tissue cyclic GMP content was investigated in the isolated guinea-pig aorta. ACh and SP relaxed aortic rings precontracted with noradrenaline (NA) or high-K solution ([K+]o=38.8 mM), in an endothelium-dependent manner. The amplitude of relaxation was larger for SP than for ACh. Nitroarginine inhibited ACh-induced but not SP-induced relaxation in NA-contraction, while this chemical inhibited both ACh- and SP-induced relaxations in high-K contraction. The tissue cyclic GMP content was not changed by nitroarginine or by removal of endothelial cells, but was elevated by stimulation with NA, ACh or SP by a factor of about 3, 5 or 11 times, respectively. These actions of ACh or SP were endothelium-dependent, and were inhibited by nitroarginine and remained unaltered by high-K solution. Thus, ACh and SP relax muscles indirectly by releasing endothelial factors, and the former by releasing mainly an endothelium-derived relaxing factor (EDRF), and the latter by releasing EDRF and other unidentified factors. As the relaxing actions of the latter factors are inhibited by high-K solution with no relation to the production of cyclic GMP, an involvement of hyperpolarizing factor, possibly EDHF, is suggested.

Key words: Aorta, Relaxation, cyclic GMP, EDRF, EDHF

Introduction

Vascular endothelial cells produce many types of vasorelaxant such as endothelium-derived relaxing factor (EDRF), hyperpolarizing factor (EDHF) and prostacyclin (PGI₂), in response to physiological, pharmacological and pathological stimuli (Furchgott, 1984; Vanhoutte et al., 1986; Moncada et al., 1991). EDRF may be nitric oxide (NO) metabolized from L-arginine or related NO-containing substances, and the EDRF-induced relaxation is accompanied by an increased production of cyclic GMP in smooth muscles (Ignarro and Kadowitz, 1987; Moncada et al., 1991). EDHF may be a metabolite of arachidonic acid through activation of an enzyme cytochrome P450 (Hecker et al., 1995), and this factor hyperpolarizes the membrane by activation of K⁺-channels, thus induces relaxation (Suzuki and Chen, 1990;
Garland et al., 1996). PGI₂ hyperpolarizes the membrane by activating ATP-sensitive K⁺-channels in coronary artery of the porcine (Siegel et al., 1992) or guinea-pig (Parkington et al., 1993) and also activates adenylate cyclase to increase the production of cyclic AMP in vascular smooth muscles (Kukovetz et al., 1979), both of them could cause muscle relaxation (Parkington et al., 1996).

The contribution of these vasodilators to the endothelium-dependent relaxation differs among tissues and also by stimulants. For example, the smooth muscle of the rabbit aorta exhibits an acetylcholine (ACh)–induced endothelium-dependent relaxation which is primarily sensitive to Nω-nitro-L-arginine (nitroarginine) indicating release of EDRF (Moncada et al., 1991). By comparison, the endothelium-dependent relaxation in the mesenteric artery is primarily nitroarginine-insensitive and sensitive to high-potassium solution (Garland et al., 1992; Garland et al., 1995). ACh–induced relaxation which is insensitive to nitroarginine but can be blocked by high-potassium solution, is produced mainly in peripheral smaller arteries (Garland et al., 1995). These observations suggest that EDHF is also involved in the endothelium-dependent relaxation. In the guinea-pig carotid artery, ACh and substance P (SP) relax muscles contracted with noradrenaline (NA), to a similar extent, but the factors involved are mainly EDRF for ACh and EDRF and EDHF for SP, since the ACh–induced relaxation is sensitive to nitroarginine whereas the SP–induced relaxation is sensitive to both nitroarginine and high-potassium solution (Zhang et al., 1994). Endothelium–derived prostanoids are also involved in the ACh–induced relaxation in the guinea-pig coronary artery (Parkington et al., 1996) or the human pulmonary artery (Zhang et al., 1996).

The guinea-pig aorta is found to be a unique, because ACh is not a potent relaxant in this artery (Hozumi et al., 1994). Therefore, the experiments were carried out to investigate the mechanisms of the ACh–induced relaxation in this tissue, by measuring mechanical responses and production of cyclic GMP. SP produced marked relaxation in this artery, in an endothelium–dependent manner (Hozumi et al., 1994). Thus, attempts were also made to compare the actions of ACh with those of SP, on the endothelium–dependent relaxation and production of cyclic GMP in this tissue. The results showed that the weak relaxing actions of ACh on the guinea-pig aorta are mainly related to the low potency to produce cyclic GMP in smooth muscles, while the potent actions of SP are related to the contribution of both EDRF and EDHF in the relaxation.

Methods

Male albino guinea-pigs (body weight, 250–300 g) were anesthetised by injecting pentobarbital sodium (30 mg/kg), and sacrificed by bleeding from the femoral artery. The thoracic aorta was isolated, and the surrounding connective tissues and fats were removed in aerated Krebs solution.

Aortic ring preparations of about 1 mm width were prepared for recording mechanical responses. In some preparations, the endothelial cells were removed mechanically by rubbing the internal surface of the vessel with moistened cotton balls, following the methods described by Furchgott and Zawadzki (1980). The successful removal of functional endothelial cells was
confirmed by the absence of any relaxing responses by SP in the NA-contracted ring preparation. An aortic ring was mounted in a recording chamber made of Lucite plate (0.5 cm width, 2 cm long, 0.5 cm depth, with the capacity about 1.5 ml). Two stainless steel wires (300 µm diameter) were inserted in parallel into the ring preparation, each from the opposite directions with one wire fixed horizontally and the other connected to a mechano-transducer (FD pick-up, Nihon Kohden TB-612T, Tokyo, Japan). The mechanical responses of circular muscles of the aortic ring were measured isometrically, using these two wires. The vessel was superfused with oxygenated Krebs solution warmed to 35.5°C, at a constant flow rate of about 3 ml/min. The tissues were incubated in unstretched condition for 1 h, and then a resting tension of 50 mg was applied to maintain the strength of muscle tension at levels similar to that in vivo. The muscle responses were displayed on a pen-writing recorder (WP-2201, National, Yokohama, Japan), after amplification (SS-210, Nihon Kohden, Tokyo, Japan).

Cyclic GMP content in tissues was measured in vessels of about 1 cm in length. Descendothelialized vessels were prepared by cutting open vessels along their long axis and rubbing the internal surface with a moistened cotton ball (Furchgott and Zawadzki, 1980). The tissues were incubated in oxygenated Krebs solution warmed to 35.5°C for over 1 h, before starting the experiments. The tissue cyclic GMP content was measured using the radio immunoassay methods reported by Moritoki et al. (1991). Briefly, the tissues were frozen quickly by soaking into liquid nitrogen, homogenized with 1 ml of 6% trichloracetic acid solution, and then centrifuged for 20 min at 3,000 × g. The supernatant was allowed to immunoreacted with cyclic GMP kits (Yamasa Shouyu, Chosi, Japan), and the cyclic GMP content was measured from the radioimmunoreactivity of the level. The concentration of protein in the precipitant of the homogenized solution was measured, according to Lowry et al. (1951). The cyclic GMP content in the tissue was expressed by pmol/mg protein.

Ionic composition of the Krebs solution was as follows (in mM); Na⁺ 137.4, K⁺ 5.9, Ca²⁺ 2.5, Mg²⁺ 1.2, HCO₃⁻ 15.5, H₂PO₄⁻ 1.2, Cl⁻ 134, glucose 11.5. The high-K solutions were prepared by replacing NaCl with KCl, isotonically (in case of 118mM [K⁺]_o solution, all NaCl was replaced with KCl). The solutions were aerated with O₂ containing 5% CO₂, and pH of the solution was maintained at 7.2-7.3.

Drugs used were acetylcholine chloride, indomethacin, noradrenaline hydrochloride (all from Sigma Chem., St. Louis, MO, USA), pentobarbital sodium (Nembutal injection, Abbott, IL, U.S.A.), Nω-nitro-L-arginine (nitroarginine) and substance P (Peptide Institute, Osaka, Japan). Indomethacin was dissolved into 5 mM Na₂CO₃ solution, at a concentration of 5 mM, and added into the Krebs solution to obtain desired concentrations (usually 5 × 10⁻⁶ M). These procedures did not detectably change the pH of the solution.

The experimental values were expressed by the mean±standard deviation (S.D.), and statistical significance was determined using Student's t-test. Probabilities of less than 5% (P < 0.05) were considered significant.
Results

Properties of the endothelium-dependent relaxation produced by ACh and SP

In isolated ring preparations of the guinea-pig aorta, application of noradrenaline (NA, $10^{-9}$–$10^{-5}$ M) produced a contraction with amplitude increased in a concentration-dependent manner, and reached the maximum value of about 1.5 times the contraction produced by 118 mM $\left[K^+\right]_o$ solution, at $10^{-5}$ M. Muscles were also contracted by high-potassium (high-K) solution ($\left[K^+\right]_o=38.8$ mM), and the amplitude was $60.0\pm5.0\%$ (n=10) of that produced by 118 mM $\left[K^+\right]_o$ solution. Contractions of comparable amplitude with those by the high-K solution were also produced by $10^{-6}$ M NA ($63.0\pm5.0\%$ of the 118 mM $\left[K^+\right]_o$-induced contraction, n=10). The experiments were, therefore, carried out in ring preparations stimulated with solutions containing either $10^{-6}$ M NA or 38.8 mM $\left[K^+\right]_o$.

Fig. 1. Endothelium-dependent relaxation of aortic ring preparation in response to ACh or substance P (SP). A and B, relaxations produced by ACh ($10^{-6}$ M) and SP ($10^{-7}$ M) respectively in ring preparations contracted with $10^{-6}$ M noradrenaline (NA). C and D, summary of the relaxation produced by ACh ($10^{-6}$ M) and SP ($10^{-9}$–$10^{-7}$ M) in rings contracted with $10^{-6}$ M NA (C) and high-K solution (D). High-K solution, $\left[K^+\right]_o=38.8$ mM. Peak amplitude of the relaxation is expressed as % of the NA- or high-K-contraction. Mean±S.D. (n=5–12).
In aortic rings precontracted with NA, application of ACh (10^{-6} M) produced a sustained relaxation (Fig. 1, A). The amplitude of relaxation increased in a concentration-dependent manner between 10^{-8}-10^{-6} M ACh and reached the maximum value of about 33% of the NA-contraction at 10^{-6} M ACh (data not shown). Application of SP also relaxed the NA-contracted aortic rings in a transient manner, with the peak amplitude of the relaxation reaching the peak at about 2 min and then subsiding during continued application of SP (Fig. 1, B). In some preparations, the transient relaxation was followed by a sustained phase with 10–20% of the peak amplitude. The peak amplitude of the SP-induced relaxation was increased in a concentration-dependent manner between 10^{-9}-10^{-7} M SP, with the maximum relaxation of about 82% of the NA-contraction produced by 10^{-7} M SP (data not shown). The relaxation of comparable amplitude to that elicited by 10^{-6} M ACh was produced by 10^{-9} M SP.

The relaxation produced by ACh or SP was not altered by indomethacin (5×10^{-6} M) (n=3, data not shown). The relaxation was also endothelium-dependent and no detectable mechanical response (relaxation or contraction) was elicited by these stimulants in tissues in which the endothelial cells had been removed (n=5).

Figure 1C summarizes the relaxation produced by ACh (10^{-6} M) and SP (10^{-9}-10^{-7} M) in aortic ring. In the presence of NA, SP relaxed the rings more than ACh. In aortic rings contracted with high-K solution, ACh or SP produced relaxing responses similar to those seen in the NA-contracted rings, but with consistently reduced amplitude (Fig. 1, D). The relaxations comparable to those produced by 10^{-6} M ACh were elicited by 10^{-8} M SP in high-K solution.

**Effects of nitroarginine on ACh- or SP-induced relaxation**

Application of 10^{-5} M nitroarginine either produced no detectable change in the resting tension (n=6), or occasionally elevated the tension by 3±2% of the NA-contraction (n=4), and

![Graphs](image-url)
Fig. 3. Effects of nitroarginine on relaxation produced by ACh or SP in aortic rings contracted with high-K solution (K\textsubscript{o}, [K\textsuperscript{+}]\textsubscript{o}=38.8 mM). Responses to ACh (10\textsuperscript{-6} M) or SP (10\textsuperscript{-7} M) were recorded from the same tissue, before (A for ACh, B for SP) and after application of 10\textsuperscript{-5} M nitroarginine (C for ACh, D for SP).

Elevated the amplitude of contractions produced by NA to 1.5-2 times the control (1.73±0.3 times, n=10). In the presence of 10\textsuperscript{-5} M nitroarginine, ACh did not, but SP did relax the NA-contracted aortic rings (Fig. 2). The mean value of the ACh-induced relaxation was reduced by nitroarginine from 25±5% to 1±2% (n=5, p<0.01), while the SP-induced relaxation remained unchanged (from 85±7% to 80±10%, n=5, P>0.05).

The effects of nitroarginine on relaxation produced by ACh or SP were also observed in aortic rings contracted with high-K solution containing 38.8 mM [K\textsuperscript{+}]\textsubscript{o}, the objective being to eliminate possible involvement of EDHF-induced hyperpolarization in the endothelium-dependent relaxation (Chen and Suzuki, 1989; Suzuki and Chen, 1990). Pretreatment with nitroarginine caused increase in amplitude of contractions produced by high-K solution to 1.8±0.5

Fig. 4. Cyclic GMP content in intact (A) and endothelium-rubbed aortic tissues (B) stimulated with ACh (10\textsuperscript{-8} M) or SP (10\textsuperscript{-7} M). Control, cyclic GMP content before stimulation. The mean value (+S.D., n=6 for each condition) of cyclic GMP content is expressed by pmol/mg protein.
times the control (n=10), and reduced the relaxation produced by ACh or SP to a negligible amplitude (Fig. 3).

Tissue cyclic GMP content elevated by ACh or SP

At rest, the amount of cyclic GMP in intact tissues of the guinea-pig aorta was 0.50 ± 0.2 pmol/mg protein (n=6), and this was increased to about 5 times by application of 10^{-6} M ACh and to 11 times by application of 10^{-7} M SP (Fig. 4A). In tissues with no functional endothelial cells by rubbing the internal surface of the vessel, the cyclic GMP content was similar to that in endothelium-intact tissues (0.45 ± 0.3 pmol/mg protein, n=5, P > 0.1), and addition of ACh or SP did not change the cyclic GMP content (Fig. 4B). In separate experiments, nitroarginine did not change the tissue cyclic GMP content (0.49 ± 0.3 pmol/mg protein, n=5, P > 0.1), and prevented the actions of ACh or SP on the production of cyclic GMP (n=5, data not shown).

Application of 10^{-6} M NA elevated the contents of cyclic GMP to about 3 times the control, and addition of 10^{-6} M ACh increased the cyclic GMP content further by about 3 times (Fig. 5). The net increase in cyclic GMP content by ACh was, therefore, enhanced by NA from about 1.5 pmol/mg protein to 3.0 pmol/mg protein (Figs. 4 and 5). In the presence of NA, the SP-induced production of cyclic GMP was also enhanced, at any given concentration (10^{-9}-10^{-7} M). Comparable amount of cyclic GMP was produced by 10^{-6} M ACh and 10^{-9} M SP (Fig. 5). In high-K solution, the amount of cyclic GMP in tissues was not significantly changed, and addition of ACh or SP elevated the cyclic GMP contents to levels similar to those seen in normal Krebs solution (compare Fig. 6 with Fig. 4). Addition of nitroarginine to the high-K solution inhibited the actions of ACh or SP to produce cyclic GMP (Fig. 6).
Discussion

In many isolated vascular tissues, ACh and SP are potent stimulants of the endothelium-dependent relaxation (Furchgott, 1984; Vanhoutte et al., 1986; Moncada et al., 1991). This is also the case in the guinea-pig carotid artery, and these agonists relax NA-contracted muscles to a similar extent, but with different mechanisms, i.e., ACh relaxes mainly by releasing EDRF and SP relaxes by releasing EDRF and EDHF (Zhang et al., 1994). The guinea-pig aorta differs from the carotid artery, in that ACh was weaker than SP in producing the endothelium-dependent relaxation. The experiments were, therefore, designed to elucidate the differences in the cellular mechanisms of the vasodilatation produced by ACh and SP in the guinea-pig aorta. As EDRF increases the tissue cyclic GMP content (Ignarro and Kadowitz, 1987; Moncada et al., 1991), the amount of EDRF contributing to the relaxation was estimated from the contents of this cyclic nucleotide in the tissue. Experiments were therefore carried out to observe the relationship between relaxation and tissue cyclic GMP content.

The present experiments showed that 1) in NA-contracted aortic rings, SP was about 3 times more potent than ACh in producing the endothelium-dependent relaxation, 2) relaxations comparable to those by $10^{-6}$ M ACh (equal to the maximum relaxation by ACh) were elicited by $10^{-9}$ M SP, 3) in high-K solution, the amplitude of relaxation was smaller than in the NA-containing solution by 30-50%, for both ACh and SP, 4) in NA-contracted aortic rings, nitroarginine markedly inhibited the ACh-induced relaxation, but had no significant inhibition on the SP-induced relaxation, 5) in ring preparations contracted with high-K solution, nitroarginine was a potent inhibitor of the endothelium-dependent relaxation produced by both ACh and SP, 6) ACh and SP increased cyclic GMP content, in an endothelium-dependent manner, and the actions were stronger for SP than for ACh, 7) NA increased cyclic GMP content and enhanced the actions of ACh, but not of SP, on the production of cyclic GMP, 8) in high-K solution, SP and ACh elevated cyclic GMP content to levels similar to that seen in the presence of NA, and 9) the stimulating actions of ACh and SP on the production of cyclic GMP were blocked by nitroarginine.

Nitroarginine inhibits the production of nitric oxide (Moncada et al., 1990), and therefore the reduction by this chemical of the relaxation produced by ACh may be due to the inhibition of EDRF production. The relaxations produced by ACh or SP in high-K solution may be also related to the production of nitric oxide, as the relaxation and production of cyclic GMP are both inhibited by nitroarginine. Thus, both ACh and SP can release EDRF from the aortic endothelial cells. However, relaxations produced by SP in muscles contracted with NA were little affected by nitroarginine. These results indicate that SP may relax muscle by releasing EDRF and nitroarginine-resistant factors. The latter apparently contributes more to the SP-induced relaxation than EDRF. The actions of the nitroarginine-resistant factor were sensitive to high-K solution, indicating that the relaxation produced by this factor is accompanied by a hyperpolarization of the membrane probably as a result of an increased K+ conductance. A similar response is also found in the SP-induced relaxation in the guinea-pig carotid artery (Zhang et al., 1994). The actions of such factor resemble to those of EDHF (Suzuki and Chen, 1890; Suzuki et al., 1990). Thus, we postulate that in the guinea-pig aorta, SP produces an
endothelium-dependent relaxation by releasing EDRF and EDHF. As the inhibitory actions of nitroarginine on the SP-induced relaxation are very weak, EDHF appears to be more potent than EDRF in producing the relaxation.

ACh is a potent stimulant for the release of EDRF and EDHF in many types of vessels (Furchgott, 1984; Vanhoutte et al., 1986; Garland et al., 1995). However, the present experiments showed that this is not the case in the guinea-pig aorta. The weak relaxing actions of ACh were related to the low production of cyclic GMP in smooth muscle, and therefore possibly due to the small levels of EDRF production. This was not an inherent property of the aortic endothelial cells, because SP could produce more cyclic GMP (equal to the larger amount of EDRF production) and more nitroarginine-resistant relaxation than ACh. Therefore, we suggest that in the guinea-pig aorta, ACh has weak actions to produce EDRF and EDHF. This is in contrast with the guinea-pig carotid artery which is relaxed by both ACh and SP to a similar extent (Zhang et al., 1994).

The reduction of the endothelium-dependent relaxation in high-K solution is partly due to the absence of EDHF-induced component of the relaxation, because EDHF cannot hyperpolarize the membrane in high-K solution (Chen and Suzuki, 1989; Suzuki and Chen, 1990; Suzuki et al., 1990). However, high-K solution reduces endothelial Ca\(^{2+}\) concentration due to absence of any voltage-activated Ca\(^{2+}\)-channel in the endothelial membrane (Laskey et al., 1990). The production of EDRF is related to endothelial Ca\(^{2+}\) concentrations (Ignarro and Kadowitz, 1987; Moncada et al., 1991), and therefore high-K solution would have inhibitory actions on the ACh- or SP-induced production of EDRF. In fact, the reduced production of EDRF in high-K solution has been confirmed in cultured endothelial (Laskey et al., 1990). In the present experiments, the low productivity of EDRF in high-K solution was also suggested indirectly from the weaker actions of the ACh- and SP-induced relaxation in muscles contracted with high-K solution than with NA. However, the production of EDRF estimated from the cyclic GMP content in tissues was not altered by high-K solution (compare Fig.4A with Fig.6A). These results could be interpreted if the reduction of the EDRF-induced relaxation by high-K solution is causally related to the depolarization of smooth muscle membrane. Alternatively, possible involvement of an increase in sensitivity of contractile protein to Ca\(^{2+}\) in smooth muscle by membrane depolarization (Okada et al., 1993) is considered.

NA has dual actions on arterial tissues, direct excitatory actions to vascular smooth muscles through activation of alpha-1 adrenoceptors and indirect inhibitory actions by elevated production of EDRF through activation of endothelial alpha-2 adrenoceptors (Cocks and Angus, 1983; Angus et al., 1986). The elevated production of cyclic GMP by NA in the guinea-pig aorta may be due to the increased release of EDRF, and this is probably related to the increase in amplitude of the NA-induced contraction by nitroarginine.

It is concluded that in the guinea-pig aorta, ACh and SP relax muscle in an endothelium-dependent manner, and the latter is more potent than the former mainly because of differences in potency to produce EDRF. Also, the differences in potency to induce relaxation between ACh and SP has causal relation to an involvement of EDHF, in addition to EDRF, and ACh relaxes mainly by releasing EDRF alone while SP relaxes by releasing both EDRF and EDHF.
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