Relatively Immediate Relaxant Effects of Cholera Toxin on Isolated Rabbit Blood Vessels

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Abstract

A study was made on the relatively immediate relaxant effect of cholera toxin (CTX) on the isolated ear artery, thoracic aorta and saphenous vein of the rabbit. Both preparations of CTX, containing sodium azide (NaN_3) and azide-free, showed no effect on the non-precontracted artery, but CTX containing NaN_3 relaxed the moderately precontracted blood vessels with methoxamine promptly, i.e., with a time course of min order. However, the immediate relaxation produced by CTX containing NaN_3 was attributed mainly to NaN_3 . Azide-free CTX, on the other hand, at 1-10 $\mu\text{g/ml}$ gradually produced concentration-dependent relaxation of the precontracted vessels. The relaxant effects of CTX on the vessels were slow and long-lasting, i.e., with a time course of 10 min order. The relaxation induced by CTX was not influenced by the removal of endothelium nor by pretreatment with 10 μM indomethacin, 3 μM atropine or 3 μM propranolol. Activation of protein kinase C by a phorbol ester inhibited the relaxant effect of CTX. These results indicate that CTX relaxes the blood vessels by directly acting on the smooth muscles, without mediation by known endogenous relaxing factor, such as endothelium-derived relaxing factor (EDRF=NO) or prostaglandin I_2 (prostacyclin) and by muscarinic receptor or β -adrenoceptor.

Key words: cholera toxin, isolated blood vessels, vasorelaxation, sodium azide.

Introduction

Cholera toxin (CTX) and sodium fluoride (NaF) have often been used as tools to analyze the intracellular signal transduction, because they increase the cellular cyclic AMP level by activating the stimulatory GTP-binding regulatory protein (Gs) coupled to adenylate cyclase (Rall and Sutherland, 1955; Birnbaumer *et al.*, 1985). In the course of study on the vascular β -adrenoceptor responses, we found that NaF relaxes the blood vessels by releasing endothelium-derived relaxing factor (EDRF=NO) and contracts the endothelium-removed vessels (Cushing *et al.*, 1990; Tsuru, 1991). Thus, NaF does not seem suitable for study on the relaxant response mediated by Gs-adenylate cyclase system in the blood vessel.

CTX, on the other hand, has been shown to produce a slow-onset and long-lived inhibition of the contractile responses to α -adrenoceptor stimulation by increasing the cyclic AMP level in some arteries (Ousterhout and Steinsland, 1981; Asano *et al.*, 1988). However, there seems no report showing that CTX produces direct relaxation of contracted blood vessels. The

present study was undertaken to characterize the relatively immediate relaxant effect of CTX on the rabbit blood vessels. The following attentions were especially paid in the present experiment : (1) How much did NaN_3 , which was contained in a CTX preparation, participate in the response to CTX with NaN_3 ? Asano and coworkers (1988) have observed unexpectedly potent antagonism of the response to norepinephrine (NE) after application of $1 \mu\text{g}/\text{ml}$ CTX for only 10 min. Because the reconstituted stock solution of CTX used contained NaN_3 , the antagonism observed was likely due to the effect of NaN_3 rather than CTX. Accordingly, they minimized with much effort, the effect of NaN_3 on the responses to NE by repeated washing. (2) Whether CTX action was mediated by prostanoid production or not? Because it has been found that CTX not only enhances prostaglandin E_2 synthesis markedly in a murine macrophage cell line (Burch *et al.*, 1988), but also amplifies prostacyclin synthesis in bovine aortic smooth muscle cells (Demolle and Boeynaems, 1989). (3) In addition, what was the effect of phorbol ester on the CTX-induced vasorelaxation? Because the activation of protein kinase C by phorbol ester has been shown to have a variety of effects on hormone-sensitive adenylate cyclase activity (Heyworth *et al.*, 1984; Bozou *et al.*, 1987).

Materials and Methods

This study was approved by the Animal Welfare Committee of Hiroshima University School of Medicine.

Male Japanese White rabbits, weighing 2–3 kg, were anesthetized with pentobarbital sodium 35 mg/kg, i.v. and exsanguinated from the common carotid artery. The ear artery, thoracic aorta and saphenous vein were dissected and placed in the chilled Krebs' bicarbonate solution. The composition of the solution (in mM) was : NaCl 119, KCl 4.7, CaCl_2 2.5, KH_2PO_4 1.2, MgSO_4 1.2, NaHCO_3 25.0 and glucose 11.1. The solution was previously aerated with a gas mixture of 95% O_2 and 5% CO_2 .

Isolated blood vessel segments were cleaned of connective tissue under a dissecting microscope and made into ring preparations of 2 mm long for the thoracic aorta and 4 mm long for the ear artery and saphenous vein. In some preparations, the endothelium was rubbed gently using a thin wooden stick and the absence of endothelium was confirmed by the lack of acetylcholine or calcium ionophore A23187-induced relaxation (Tsuru, 1991). Each preparation was suspended in a tissue bath of 10 ml which contained Krebs' solution aerated with the gas mixture and maintained at 37°C as described previously (Tsuru *et al.*, 1990). The preparation was given an optimum load of 2 g in the artery and 0.3 g in the vein, and equilibrated for 1.5 hr before starting experiments. During the equilibration period, the bath solution was renewed every 15–20 min. Isometric tension was recorded on an ink-writing oscillograph (Nihon Kohden Kogyo, Tokyo, Japan, model WI-641G) via force-displacement transducers (Nihon Kohden Kogyo, Tokyo, Japan, model TB-611T).

Relaxation responses of blood vessels were examined after moderate contraction with 0.1 – $1 \mu\text{M}$ of NE or methoxamine, which produced 25–60% of NE-induced maximum contractions, to observe relaxation responses clearly. The maximum relaxation of each preparation was obtained by addition of $100 \mu\text{M}$ papaverine at the end of experiment.

Two kinds of CTX preparations, i.e., one containing sodium azide (NaN_3) and the other of

azide-free, were purchased from List Biological Laboratories, Inc. (Campbell, CA, U.S.A.). When a lyophilized powder of the former CTX preparation was reconstituted to 1 ml with distilled water, it contained 1.0 mg of the protein in Tris buffer (50 mM Tris, 1 mM Na₂EDTA, 3 mM NaN₃ and 200 mM NaCl, pH 7.5). Both the CTX stock solutions, containing NaN₃ and azide-free, at this concentration were stored at 4°C. The vehicle solution containing 50 mM Tris, 1 mM Na₂EDTA and 200 mM NaCl was also reconstituted for checking the effect of vehicle. When the action of NaN₃ was studied, NaN₃ was dissolved with the similar constitution of the vehicle solution.

(-)-Norepinephrine bitartrate, (±)-propranolol hydrochloride, indomethacin, acetylcholine chloride, atropine sulphate, calcium ionophore A23187, phorbol 12-myristate 13-acetate and 4 α -phorbol-12, 13-didecanoate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Papaverine hydrochloride was obtained from Tokyo Kasei (Tokyo, Japan).

Results shown in the text, tables and figures are expressed as the mean $\pm$ S.E.M. obtained from 4 to 6 experiments. Significance of difference between two means was determined by unpaired Student's *t*-test. Differences with *P* values less than 0.05 were considered to be statistically significant.

Results

CTX preparations, one containing NaN₃ and the other being azide-free, showed no apparent effect on the non-precontracted blood vessels. Therefore, the relaxant effects of CTX were studied using vessels precontracted moderately with 0.1-1 μ M of NE or methoxamine, which produced 25-60% of the NE-induced maximum contraction, so that the relaxant response could be clearly observed (Tsuru *et al.*, 1990).

CTX containing NaN₃ relaxed promptly the blood vessels which had been precontracted moderately with NE or methoxamine, i.e., with a time course of min order (Fig. 1A). On the other hand, NaN₃, which was dissolved in a similar constituent of CTX preparation except for CTX, relaxed the vessels similarly to CTX with NaN₃ (Fig. 1B). Based on the concentration-

Fig. 1. Representative tracing showing the prompt relaxant responses to cholera toxin (CTX) with NaN₃ (A) and to NaN₃ alone (B). Concentrations of NaN₃ (μ M) and CTX (μ g/ml) in panel A were as follows. 1: 0.1 and 0.03; 2: 0.3 and 0.1; 3: 1 and 0.3; 4: 3 and 1; 5: 10 and 3, respectively. Concentrations of NaN₃ (μ M) in panel B were as follows. 1: 0.1; 2: 0.3; 3: 1; 4: 3; 5: 10, respectively. Thus, the numbers are comparable between panel A and panel B regarding concentrations of NaN₃. Pap 100: papaverine 100 μ M.

response curves for NaN_3 with CTX and for NaN_3 alone, the pD_2 values, negative logarithms of median effective concentration, of NaN_3 were obtained as 5.79 ± 0.14 ($n=5$) in the former and 6.10 ± 0.17 ($n=5$) in the latter, respectively. There was no significant difference between these values, indicating that the effect of CTX with NaN_3 is mainly due to NaN_3 . Therefore, the following experiments were done by using an azide-free CTX preparation.

CTX at 1–10 $\mu\text{g/ml}$ gradually produced concentration-dependent relaxation of the vessels precontracted moderately with NE or methoxamine. The relaxant effects of CTX on the rabbit blood vessels were slow and long-lasting, i.e., with a time course of 10 min order (Fig. 2). The onset of CTX-induced relaxation seemed to be faster in the thoracic aorta and saphenous veins (data not shown) than in the ear artery (compare Fig. 2 and Fig. 5A). The CTX concentration-relaxant response curves in the thoracic aorta were not significantly different between the endothelium-intact and endothelium-rubbed preparations (Fig. 3), indicating that the CTX-induced relaxation was not dependent on the endothelium. As azide-free

Fig. 2. Typical recording of the relaxant effect of cholera toxin (CTX) in the rabbit thoracic aorta. The preparation was precontracted by methoxamine 0.5 μM . CTX 1, 3, 10: cholera toxin 1, 3 and 10 $\mu\text{g/ml}$. Pap 100: papaverine 100 μM .

Fig. 3. The concentration-response curve for cholera toxin in the rabbit thoracic aorta. Closed circles, control (endothelium-intact, $n=5$). Open circles, endothelium-rubbed preparation ($n=5$). Maximum relaxation was obtained by papaverine 100 μM .

CTX solution contained a cation chelating agent Na_2EDTA , Tris and NaCl, only small inhibitory responses were observed with an equivalent volume of vehicle. Namely, the relaxation produced by vehicle was less than 10% of that by CTX with vehicle, being clearly discriminable (data not shown).

The relaxation induced by $5\text{ }\mu\text{g/ml}$ CTX was not influenced by pretreatment with $10\text{ }\mu\text{M}$ indomethacin, $3\text{ }\mu\text{M}$ propranolol or $3\text{ }\mu\text{M}$ atropine (Fig. 4). The results indicate that CTX relaxes the blood vessels neither by releasing prostanoids nor by activating β -adrenoceptors and muscarinic receptors.

Phorbol 12-myristate 13-acetate (PMA) $1\text{ }\mu\text{M}$ inhibited the relaxant effect of CTX on the artery. In contrast, 4α -phorbol-12, 13-didecanoate (4α -PDD), which is a negative control of PMA, had no influence on the CTX-induced relaxation (Fig. 5). The result indicates that the activation of protein kinase C by phorbol ester inhibits the relaxant effect of CTX.

Fig. 4. Effects of atropine, propranolol and indomethacin on the cholera toxin (CTX)-induced relaxation in the ear artery. A. control, $5\text{ }\mu\text{g/ml}$ of CTX alone. B, C and D: pretreated with $3\text{ }\mu\text{M}$ atropine, $3\text{ }\mu\text{M}$ propranolol and $10\text{ }\mu\text{M}$ indomethacin, respectively. These inhibitors were administered 30 min before CTX. Pap 100: papaverine $100\text{ }\mu\text{M}$.

Fig. 5. Inhibition of relaxant response to 3 and 10 $\mu\text{g/ml}$ of cholera toxin (CTX) by activation of protein kinase C in the rabbit ear artery. A: control. B and C: pretreated with phorbol 12-myristate 13-acetate (PMA) 1 μM and its negative control, 4 α -phorbol-12,13-didecanoate (4 α -PDD), respectively, 30 min before administration of CTX.

Discussion

Both NaF and CTX have been extensively used in biochemical analyses of the β -adrenoceptor-Gs-adenylate cyclase system (Rall and Sutherland, 1958; Birnbaumer *et al.*, 1985). The present study, however, revealed that the modes of action of NaF and CTX on the blood vessels were evidently different. The most important difference is that while the NaF-induced vasorelaxation is dependent on the endothelium (Cushing *et al.*, 1990; Tsuru, 1991), the CTX-induced relaxation is not. In addition, though it has been previously shown that NaF exhibits the complex effects on the blood vessels (Zeng *et al.*, 1989), CTX is more suitable or simpler than NaF for studying the intracellular signal transduction mechanism of β -adrenoceptor-Gs-adenylate cyclase system in the blood vessels.

When Asano and coworkers (1988) used CTX to examine whether there was reduction of Gs function in the femoral artery of spontaneously hypertensive rats, they were unexpectedly confronted with a problem that the reconstituted stock solution of CTX used contained NaN_3 , which is known to be a potent activator of guanylate cyclase (Waldman and Murad, 1987). Thus, they had to eliminate the influence of NaN_3 on α -adrenoceptor-mediated contractions

with much efforts by repeated washing. In addition, they had to incubate the arterial preparation with $1\text{ }\mu\text{g/ml}$ of CTX for 1 hr to inhibit α -adrenoceptor-mediated contractions. The present study reconfirmed potent relaxant effects of NaN_3 on the blood vessels. As shown in Fig. 1, the relaxant responses to CTX with NaN_3 and to NaN_3 alone seemed to be similar on the basis of NaN_3 concentration. In fact, the pD_2 values for NaN_3 calculated from the concentration-response curves for NaN_3 with CTX and for NaN_3 alone were not different. The symbol number 5 in Fig. 1A at which the maximum relaxation was induced referred to $3\text{ }\mu\text{g/ml}$ CTX and $10\text{ }\mu\text{M}$ NaN_3 . In comparing the time courses of relaxant effect of this and of $3\text{ }\mu\text{g/ml}$ azide-free CTX shown in Fig. 5A, the notion that the relaxation induced by a mixture of CTX and NaN_3 is mainly due to NaN_3 is further strengthened. The relaxation induced by NaN_3 may be mediated by the increase in level of cyclic GMP as shown by Katsuki and collaborators (1977) in some smooth muscles.

In contrast to the prompt responses to azide-containing CTX, azide-free CTX produced slow-onset and long-lasting relaxation of the blood vessels; this action was clearly discriminable from that of the vehicle. β -Adrenoceptors and muscarinic acetylcholine receptors did not seem to be involved in the CTX-induced vasorelaxation because propranolol and atropine had no effect on it (Fig. 5). Also, prostanoids may not be involved in this phenomenon despite the earlier finding that CTX not only enhances prostaglandin E_2 synthesis markedly in murine macrophage cell line (Burch *et al.*, 1988) but also amplifies prostacyclin synthesis in bovine aortic smooth muscle cells (Demolle and Boeynaems, 1989). The present result that propranolol and indomethacin did not affect the CTX-induced vasorelaxation is consistent with that of Ousterhout and Steinsland (1981). These results indicate that CTX relaxes the blood vessels by directly acting on the smooth muscle cells.

Ousterhout and Steinsland (1981) studied the effects of CTX on the vasoconstriction induced by electrical stimulation of periarterial adrenergic nerves and by exogenously administered NE using the perfusion technique of isolated rabbit ear artery. In this preparation, when CTX was added to the perfusion fluid for 10–20 min, the α -adrenoceptor-mediated constriction began to decline after 1 to 2 hr and was gradually inhibited until pressure responses after 10 hr were less than 10% of those before toxin treatment. In addition, they described that the accumulation of cyclic AMP in arteries exposed to CTX ($4.5\text{--}9\text{ }\mu\text{g/ml}$) for 1 hr became evident only after a latent period of 1 to 2 hr and was maintained for at least 6 hr. The slow onset and long duration of action of CTX on isolated blood vessels in the present study is consistent qualitatively with the time course of action of the toxin in their experiment (Ousterhout and Steinsland, 1981), but is somewhat different quantitatively. The main difference may be due to the experimental methods employed. Regarding the mechanisms of action of CTX on blood vessels, whereas the increase in cyclic AMP level does not become evident until 2 hr after incubation with CTX in the rabbit ear artery as mentioned above (Ousterhout and Steinsland, 1981), the present study revealed that the relaxant response to CTX appeared within several ten minutes and reached the maximum within 1.5–2 hr after exposure to CTX (Fig. 4). Therefore, further investigation is needed to clarify whether cyclic AMP or some other mechanism is responsible for the relaxant effect of CTX.

PMA $1\text{ }\mu\text{M}$ inhibited the relaxant effect of CTX on the artery, whereas 4α -PDD, which is

a negative control of PMA, had no influence on the CTX-induced relaxation (Fig. 5). The result indicates that the activation of protein kinase C by phorbol ester inhibited the relaxant effect of CTX. It has been recently reported that phorbol esters, presumably acting via protein kinase C, can have a variety of effects on hormone-sensitive adenylate cyclase. For example, Heyworth and coworkers (1984), on one hand, have suggested that PMA exerts its inhibitory effect on the CTX-stimulated adenylate cyclase activity through the action of protein kinase C in rat hepatocytes, but on the other hand, Bozou and collaborators (1987) have reported that PMA fails to attenuate the action of CTX to stimulate cyclic AMP production in HT29 cell line which is derived from a human colon adenocarcinoma.

In conclusion, CTX slowly relaxes the isolated blood vessel by directly acting on vascular smooth muscle cells, though the precise action mechanism remains to be resolved.

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