Involvement of Cyclic AMP in Vasodilatation by Amrinone: A Comparative Study with 3-Isobutyl-1-Methyl-Xanthine (IBMX)

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Abstract—We investigated the effect of amrinone, a non-glycosidic, non-adrenergic cardiotonic drug, on rabbit aortic strips and vascular smooth muscle cells cultured from rabbit aorta. Amrinone relaxed the strips precontracted by KCl, norepinephrine, serotonin or STA_2 in a dose-dependent manner, and it shifted the dose-response curves downward. A similar mode of relaxation was noted with IBMX, an inhibitor of cyclic AMP phosphodiesterase (cAMPDE). Although propranolol did not affect the relaxation induced by amrinone, W-7, a calmodulin antagonist, slightly potentiated and IBMX attenuated the response. In intact smooth muscle cells in culture, amrinone increased basal levels of cAMP and markedly potentiated cAMP accumulation in response to 10^{-6} M isoproterenol. The effect on cAMP accumulation mimicked that of IBMX but the effects were not additive. Inhibition of cAMPDE was also demonstrated in a cell-free system, IC_{50} values for amrinone and IBMX being 2.1×10^{-5} M and 1.2×10^{-6} M, respectively, using a preparation of cAMPDE partially purified from the cells by DEAE cellulose chromatography. Amrinone seems to exert a direct effect on vascular smooth muscle cells by potently inhibiting cAMPDE. This inhibition would to some extent explain the vasodilatory property.

Amrinone is a positive inotropic agent which favorably alters hemodynamics in patients with congestive heart failure (1-6). Along with its inotropic action, the vasodilatory property of amrinone may contribute to the increase in cardiac output in these patients (1, 2, 6). Although the mechanism of inotropic action of the agent has been widely discussed (7-10), factors related to the properties of vasodilatation are poorly understood.

The aim of the present study was to investigate the effect of amrinone on cAMP metabolism in vascular smooth muscle cells in relation to vasodilatation. Firstly, we characterized the mode of amrinone-induced vasodilatation by monitoring the isometric tension of rabbit aortic strips in response to various agonists. The vasodilatory effect was compared with that of IBMX. Secondly, we used cultured smooth muscle cells from rabbit aorta to investigate the involvement of cAMP

metabolism in the mechanism of vasodilatation induced by amrinone or IBMX. Our results suggested that cAMP phosphodiesterase (cAMPDE) inhibition in the smooth muscle cells is involved in the mechanism of the vasodilatory property of amrinone. This is in accord with findings in cardiac muscle cells that the inhibition of this enzyme was responsible for its positive inotropism (10).

Materials and Methods

Experiments using strips of the rabbit aorta

Eighteen male albino rabbits aged 4-6 months weighing 2.8 ± 0.1 kg (Mean $\pm$ S.D.) were used. The thoracic aorta was quickly removed, and six helically cut strips of 2.5 cm $\times$ 0.5 cm were prepared from each aorta. After removing the adventitia using fine scissors, the strips were fixed vertically between hooks under a resting tension of 5 g in a 40 ml organ bath containing Hanks' solution (137 mM Na^+ , 5.7 mM K^+ , 0.9 mM

Mg²⁺, 1.3 mM Ca²⁺, 145.8 mM Cl⁻, 27.4 mM HCO₃⁻, 0.4 mM HPO₄⁻ and 5.6 mM glucose; Nissui Pharmaceutical Co., Tokyo), maintained at 37°C and aerated with a mixture of 95% O₂ and 5% CO₂ (pH 7.2–7.4). Hooks anchoring the upper end of the strips were connected to the lever of a force displacement transducer (FD pick-up TB 611T, Nihon Kohden Co., Tokyo), and the isometric tension developed was recorded on an ink-writing oscillograph (WI 640G, Nihon Kohden Co., Tokyo), as previously described (11–13).

Vasodilatory properties of amrinone were characterized by its effect on the tension of the strips precontracted by the following agonists: KCl, norepinephrine, serotonin or STA₂ (9,11-epithio-11,12-methano-TXA₂: a stable thromboxane A₂ analogue). Contractions induced by these agonists reached a peak and maintained a plateau for a period long enough to observe the effect of the relaxant. Relaxation by amrinone was expressed as percentage of the maximum relaxation induced by 10⁻⁴ M papaverine (11). Cumulative dose-response curves of the agonists (KCl, norepinephrine, serotonin, angiotensin II) were obtained in the presence of varying concentrations of amrinone or IBMX added 20 min prior to the application of the agonists. The concentration of each relaxant for 50% inhibition of maximum contraction (IC₅₀ values) by the agonists was estimated from a plot of percent inhibition of contraction vs. log concentration of the relaxant. Synergism of amrinone with the other muscle relaxants, propranolol, *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulfonamide (W-7) and IBMX, was also investigated by determining the effects of pretreatment of these relaxants on amrinone-induced relaxation of the strips precontracted by 20 mM KCl. Phentolamine, 10⁻⁶ M, was applied 20 min prior to the treatment with W-7 (See Discussion.).

Experiments using cultured vascular smooth muscle cells

Vascular smooth muscle cells from rabbit aorta were cultured according to the method of Ross (14). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10%

(V/V) fetal bovine serum (FBS, GIBCO) with the addition of penicillin (100 IU/ml) and streptomycin (100 µg/ml) (GIBCO). The medium was changed twice a week, and the cells which had undergone fewer than 10 passages were used for the following studies.

1) Effect of amrinone on cAMP accumulation in intact cells: Effect of amrinone on cAMP accumulation in the cultured smooth muscle cells was investigated using the adenine prelabeling method (15–17). The cells, grown to confluence in a 60×15 mm plastic dish (Falcon 3002), were incubated at 37°C in DMEM with [³H]-adenine at the concentration of 0.2 µM (2.2 µCi/dish) for 3 hr. Incorporation of [³H]-adenine reached a peak in 3 hr, and a plateau was maintained for at least 1 hr (peak values: 65.91±7.61%, Mean±S.D.; N=10) (17). The labeled cells were rinsed with 3 ml of 0.9% saline four times to remove the unincorporated [³H]adenine. Cyclic AMP accumulation in the cells in response to amrinone was studied in terms of the time course, dose-responsiveness and effects of *l*-isoproterenol. Incubations were terminated by aspirating off the media and adding 1 ml of ice-cold 0.4 M perchloric acid. The cells were then harvested by scraping with a rubber policeman, sonicated for 20 sec with a "low" setting of 50 on a Ohtake sonicator (Ohtake Co. Ltd., Japan), and centrifuged at 3,000 g for 10 min. cAMP in the cells was isolated by applying an aliquot of the supernatant to the AG 50WX-4 column followed by a neutral alumina oxide column, as described by Salomon (18). Recovery of the columns was monitored by addition of [¹⁴C]cAMP (2,500 dpm/sample) before application to the column. The usual recoveries of [¹⁴C]-cAMP after alumina were 65–75%.

Radioactivities were determined by a Packard Tri-carb 460 CD. cAMP accumulation in the cells was expressed as radioactivity of [³H]cAMP relative to that of the total [³H]adenine incorporated into the cells (% conversion) (16).

2) Effect of amrinone on cAMP/PDE activity: cAMP/PDE of the homogenates of the smooth muscle cells were partially purified by DEAE cellulose chromatography (19, 20). The cells grown to confluence in a

75 cm² flask (Corning) were harvested by a rubber policeman, resuspended in homogenizing buffer (10 mM Tris/Cl, pH 8.0; 50 mM NaF, 20 μ M N-p-tosyl-L-lysine chloromethyl ketone (TLCK); 20 μ M leupeptin and 1% Triton x-100 (V/V)) and homogenized in a glass homogenizer with a Teflon pestle using 10 strokes by hand at 0°C. After centrifugation (40,000 g, 30 min at 4°C), the supernatant was applied to a small column (5.5 $\times$ 100 mm) containing about 1.0 ml of DE 52 equilibrated with 15 mM sodium acetate, 5 mM 2-(N-morpholino) ethane-sulfonic acid (MES), 30% ethylene glycol (V/V), 0.1 mM dithiothreitol (DTT), 10 μ M TLCK, 10 μ M leupeptin. The column was washed by 3 bed volumes of equilibrating buffer and further washed by 5 bed volumes of the buffer with a sodium acetate concentration of 200 mM. cAMP PDE was eluted with 1.5 ml of the buffer with a sodium acetate concentration of 500 mM. This eluate contained about 50% the total PDE activity and was used as the partially purified "low K_m " cAMP PDE. The activity of cAMP PDE was assayed with the substrate concentration of 0.25 μ M according to Thompson and Appleman (21). The IC₅₀ value of amrinone and IBMX required to inhibit cAMP PDE was estimated from a plot of percent inhibition of the enzyme activity vs. log concentration of amrinone. Tissue protein was measured by the method of Lowry et al. (22) with human serum albumin as a standard.

Reagents

[2,8-³H] adenine (specific activity: 14.7 Ci/mmol), [2,8-³H] cAMP (31.5 Ci/mmol), [8-¹⁴C] cAMP (51.4 mCi/mmol) were purchased from New England Nuclear. The following materials were purchased: AG 50 WX-4 (Bio-Rad), neutral alumina oxide (Merck), DE 52 (Whatman), *l*-isoproterenol, *d,l*-propranolol, Tris, NaF, leupeptin, TLCK, IBMX, DTT (Sigma), phentolamine·HCl (Ciba), Triton X-100 (Kanto Kagaku, Japan), STA₂ (Ono Yakuhin, Japan), W-7 (Seikagaku Kogyo, Japan). Amrinone was a generous gift from Prof. S. Shibata of Hawaii University, U.S.A. All the other reagents were of the best grades available and were not purified further.

Results

Amrinone-induced relaxation of rabbit aortic strips: Amrinone elicited a dose-dependent relaxation of the aortic strips precontracted by all the agonists tested, as shown in Fig. 1. Amrinone at 4×10^{-4} M relaxed the strips exposed to 20 mM KCl, 5×10^{-7} M norepinephrine, 10^{-6} M serotonin and 10^{-7} M STA₂ by 57.9%, 88.9%, 84.9% and 26.7%, respectively (Values are the means of triplicate determinations).

Preincubation with amrinone for 20 min before application of the agonists shifted the dose-response curves for KCl, norepinephrine, serotonin or angiotensin II significantly downward and slightly to the right (Fig. 2). Contractions by angiotensin II were most susceptible to amrinone followed by serotonin, norepinephrine and KCl. IC₅₀ values of amrinone for inhibition of maximal

Fig. 1. Relaxing effect of amrinone on contractions induced by agonists. Following a 2 hr equilibration period, the vasoconstricting agents were added to the medium (arrows). After the contraction reached a plateau, amrinone was applied cumulatively (dots). Finally, 10^{-4} M papaverine was applied to obtain maximum relaxation (11).

contraction by KCl, norepinephrine, serotonin and angiotensin II were calculated to be $>10^{-3}$ M, 5.2×10^{-4} M, 7.3×10^{-5} M and 2.9×10^{-5} M, respectively. Similarly, IBMX shifted the dose-response curves downward (Fig. 3), although IBMX proved to be a more potent vasodilator, IC_{50} values being 6.4×10^{-4} M, 4.0×10^{-5} M, 2.8×10^{-5} M and 1.1×10^{-5} M, respectively (Values are

derived from 4–6 separate experiments).

Effects of pretreatment with propranolol, W-7 or IBMX on amrinone-induced relaxation are shown in Fig. 4. As compared with the control tracings, propranolol had no effect, and W-7 slightly potentiated the relaxation by amrinone. Pretreatment with IBMX, however, appeared to attenuate the response, although both W-7 and IBMX

Fig. 2. Effect of amrinone on dose-response curves of the agonists. The strips were treated with 10^{-5} – 10^{-3} M amrinone for 20 min, and the agonists were applied cumulatively. Maximum contractions by each agonist were taken as 100%. Values are the means $\pm$ S.E.M. of 4–6 experiments.

inhibited 20 mM K^+ -induced contraction by about 30%.

cAMP accumulation in intact smooth muscle cells in culture: The time course of cAMP accumulation in the cells after application of 10^{-3} M amrinone is shown in Fig. 5. Incubation with amrinone significantly increased the cAMP levels at all incubation periods, being highest at 1 min, maintaining a plateau after 5 min. The cAMP levels in

the cells incubated without amrinone were virtually unchanged throughout. Amrinone induced dose-dependent increase in cAMP accumulation both in the basal levels (Table 1) and in the response to 10^{-6} M isoproterenol (Fig. 6). It is noteworthy that cAMP accumulation in response to isoproterenol was markedly potentiated in the presence of amrinone.

IBMX at 10^{-3} M had a similar effect on

Fig. 3. Effect of IBMX on the dose-response curves of the agonists. The experimental protocol was the same as in Fig. 2.

cAMP accumulation, but combined treatment with 10^{-3} M amrinone did not differ significantly in terms of cAMP accumulation (Fig. 7).

Fig. 4. Effect of pretreatment with propranolol, W-7 or IBMX on amrinone-induced relaxation. These agents were added to the medium 20 min prior to KCl (20 mM) application. Phentolamine, 10^{-6} M, was applied 20 min prior to the treatment of W-7. After the contraction by KCl reached a peak, amrinone was applied cumulatively. Each tracing was recorded after 2 hr of equilibration following the control tracing (left side of the panel). W indicates washing of the strip with the medium.

Fig. 5. Time course of cAMP accumulation in the cells incubated in the presence or absence of 10^{-3} M amrinone ($N=4$). The $[^3H]$ -labeled cells were rinsed four times with 3 ml of 0.9% saline and incubated with the medium.

Effect of amrinone on cAMP/PDE: The activity of a partially purified preparation of cAMP/PDE from the cultured smooth muscle cells was 0.8 nmol/min/mg protein, as determined by the substrate concentration of $0.25 \mu\text{M}$. Dose-inhibition curves for amrinone and IBMX are shown in Fig. 8. Amrinone inhibited the enzyme in a dose-dependent manner with an IC_{50} value of 2.1×10^{-5} M; and at a concentration of 10^{-3} M, complete inhibition was achieved. IBMX also inhibited the enzyme in a similar fashion, but appeared

Table 1. Dose-responsiveness of cAMP accumulation in the cells incubated with amrinone

	cAMP (% conversion)
Control	0.329 ± 0.017
10^{-6} M Amrinone	0.351 ± 0.028
10^{-5} M Amrinone	0.365 ± 0.012
10^{-4} M Amrinone	$0.523 \pm 0.029^*$
10^{-3} M Amrinone	$0.848 \pm 0.046^*$

Values are the means $\pm$ S.E.M. of 4 dishes. *Significantly different from the control cells ($P < 0.01$).

Fig. 6. Effect of amrinone on cAMP accumulation in response to isoproterenol. After preincubation in the absence (control) or presence of amrinone (concentrations indicated) for 20 min, 10^{-6} M isoproterenol was applied to the incubation medium at 0 min, and the time course of cAMP accumulation was observed (Mean $\pm$ S.E.M.; $N=4-8$).

Fig. 7. Effect of IBMX and amrinone on cAMP accumulation and their interaction. The cells were treated with 10^{-3} M amrinone and/or 10^{-3} M IBMX at 20 min prior to application of 10^{-6} M isoproterenol, and cAMP accumulation was compared with the control cells without pretreatment before isoproterenol.

to be more potent than amrinone with an IC_{50} value of 1.2×10^{-6} M.

Discussion

Meisheri et al. (23) reported that amrinone relaxed rabbit aortic rings precontracted by high K^+ and norepinephrine and that this drug increased cAMP concentration in the rings. They postulated that the inhibitory effect of amrinone on the contraction appeared to be nonspecific and might be related with cAMP metabolism in the smooth muscle cells. Our results support their conclusion in that amrinone acted as a nonspecific vasodilator which relaxed all the strips precontracted by various contractile agents and shifted the dose-response curves downward and in a dose-dependent manner. A similar mode of relaxation was noted with IBMX.

Effects of W-7 and IBMX on relaxation by amrinone (Fig. 4) are of special interest. W-7

Fig. 8. Inhibition of cAMP PDE activity by amrinone and IBMX ($N=3$).

was confirmed to have at least dual pharmacological actions: calmodulin-related relaxation and calmodulin-independent contraction (24). Since our purpose of using W-7 was to compare the mode of relaxation with those of other relaxants, the latter action should be minimized. This was achieved by treating the arterial strip with 10^{-6} M phentolamine at 20 min prior to the application of amrinone, because the mechanism of calmodulin-independent contraction involved increased efflux of norepinephrine from sympathetic nerve terminals (24). Phentolamine at this concentration did not affect K^+ -induced contraction or amrinone-induced relaxation significantly (data not shown). Pretreatment with 5×10^{-5} M W-7 or 10^{-5} M IBMX inhibited 20 mM K^+ -induced contraction of rabbit aorta by 30%, but W-7 enhanced relaxation by amrinone, while IBMX failed to elicit an additive effect. W-7, in addition to the calmodulin-related relaxation, was reported to inhibit cAMP PDE (25). Thus, enhancement of relaxation by W-7 could be explained by inhibition of cAMP PDE. However, as noted by Hidaka et al., the IC_{50} value for inhibiting "low K_m "

cAMPPDE, a partially purified preparation from human vascular smooth muscle cells, was 320 μ M (25), which is much higher than that of IBMX. Therefore, it seems unlikely that inhibition of cAMPPDE by W-7 is responsible for the additive effect. Conclusively, it is suggested that W-7 enhances relaxation by amrinone probably through interaction with calmodulin and that a common mechanism is involved in the relaxation by amrinone and IBMX.

Our current study using rabbit aortic strips suggested amrinone relaxed the arterial wall probably through direct inhibition of smooth muscle cells, possibly mediated by cAMPPDE inhibition. To further characterize the effect of amrinone on vascular smooth muscle cells, we turned to the cell culture technique, employing smooth muscle cells from rabbit aorta. These cells retained thin and thick filaments, responded to catecholamines as well as phosphodiesterase inhibitors and were found to be a good model system in which to study cAMP metabolism (17). Furthermore, this approach could eliminate some of the heterogeneities inherent to vessel walls such as effects of endothelium, connective tissue or uneven distribution of nervous systems.

We confirmed that 10^{-3} M amrinone increased cAMP levels about 2.5-fold and markedly potentiated cAMP accumulation, in response to 10^{-6} M isoproterenol in cultured smooth muscle cells. Although IBMX had similar effects on cAMP accumulation, the combined treatment added little to the increase in cAMP accumulation at basal levels and in response to isoproterenol. These results again suggested that amrinone, like IBMX, enhanced cAMP accumulation through inhibition of cAMPPDE in intact smooth muscle cells. Amrinone inhibited the partially purified low K_m cAMPPDE with an IC₅₀ value of 2.1×10^{-5} M. IBMX was a more potent inhibitor with an IC₅₀ value of 1.2×10^{-6} M. It is noteworthy that IC₅₀ values for inhibition of cAMPPDE by the two relaxants appeared to correlate with those for inhibition of contraction by the agonists. Endoh et al. (10) reported that amrinone enhanced cAMP accumulation in isolated canine ventricular muscle through inhibition

of cAMPPDE and that the levels of cAMP in the specimen positively correlated with the inotropism of amrinone. They also showed that amrinone inhibited the partially purified preparation of cAMPPDE with an IC₅₀ value of 3 to 4×10^{-5} M, such being similar to the findings in our study. Thus, amrinone may produce concomitantly a positive inotropism and vasodilatation, through a common mechanism: inhibition of cAMPPDE.

Clinical reports revealed that increases in the cardiac index showed a linear relationship to the plasma concentration of amrinone in a range of 0.5 μ g/ml to 7 μ g/ml, i.e., 2.7×10^{-6} M to 3.7×10^{-5} M. (3), such being comparable with the concentration at which amrinone exhibited a pharmacological action in the present studies. Moreover, it has been shown that amrinone improves hemodynamic indices by vasodilatation with no evidence of a direct effect on contractility in patients with chronic heart failure (26). The amrinone-induced alteration in cAMP accumulation in vascular smooth muscle cells may play an important role in the therapeutic effects of this agent.

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