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SELECTIVE INHIBITION OF CYCLIC AMP PHOSPHODIESTERASE FROM VARIOUS HUMAN TISSUES BY MILRINONE, A POTENT CARDIAC BIPYRIDINE

MASAAKI ITO, TOSHIO TANAKA,* MASAHIRO SAITOH,* HIROSHI MASUOKA, TAKESHI NAKANO and HIROYOSHI HIDAKA*†

First Department of Internal Medicine, and * Department of Molecular and Cellular Pharmacology, Mie University School of Medicine, 2-174 Edobashi, Tsu, Mie 514, Japan

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Abstract—We observed the effects of milrinone, an inotropic agent prescribed to treat congestive heart failure, on cyclic nucleotide messenger systems in various human tissues *in vitro*. Cyclic nucleotide phosphodiesterases (PDEs) from the human heart were separated into three isoforms, FI, FII and FIII, by DEAE-cellulose chromatography. Milrinone proved to be a potent and selective inhibitor of human cardiac FIII PDE, a "low K_m" enzyme for cyclic AMP (cAMP-PDE). The IC_{50} value for the inhibition of FIII PDE was 0.42 μM, while those of FI and FII PDEs, "high K_m" enzymes, were 38 and 19 μM, respectively. Kinetic studies showed that milrinone inhibited the activity of FIII PDE, competitively with respect to cAMP, and the K_i was 0.15 μM. Milrinone in doses to 100 μM had no effect on human cardiac cAMP-dependent protein kinase and adenylate cyclase. The activity of cAMP-PDEs from human platelets and the aorta, as well as that from heart, were potently inhibited by milrinone, with much the same IC_{50} values. Cyclic AMP-PDEs from human kidney, liver and lung were not readily inhibited by milrinone, and the IC_{50} values of cAMP-PDEs from these tissues were about 7–30-fold higher than that from heart. On the other hand, papaverine had a relatively lesser selectivity for any of the cAMP-PDEs. All these results suggest that milrinone exerts inotropic effects by inhibiting cAMP-PDE selectively in the human heart tissues and that this compound can be used to evaluate different forms of cAMP-PDEs present in human tissues.

Milrinone is a potent cardiac bipyridine with inotropic and vasodilator properties [1] and is prescribed clinically. This drug increases the cardiac contractile force, maximum left ventricular development and cardiac output with minimal change in heart rate [1]. It has been suggested that the pharmacology of bipyridine derivatives, such as milrinone and amrinone, results from an inhibition of a "low K_m" cAMP phosphodiesterase (cAMP-PDE) and increases cardiac cAMP levels in various species [1–5]. Recent biochemical studies revealed that variations of cAMP-PDEs with respect to size, substrate specificities, effects of drugs and immunological aspects in various tissues of several species [3, 6–11]. Such being the case, we investigated the biochemical effects of milrinone on phosphodiesterase from the human cardiac tissues and compared the findings with those from other tissues.

MATERIALS AND METHODS

Cyclic nucleotide phosphodiesterase (PDE) was partially purified from various human tissues, including heart (ventricle), aorta, lung, liver and kidney, by DEAE-cellulose chromatography, as described [12, 13]. These tissues were obtained within 2 hr of autopsy on a 54-year-old Japanese woman who died of a cerebrovascular accident. The excised tissues were washed in saline and stored at −80°. Human platelet phosphodiesterase was prepared as described [14]. Cyclic nucleotide phosphodiesterases were purified from human platelets by sonication, centrifugation at 105,000 *g* and DEAE-cellulose chromatography. Calmodulin (CaM) was isolated from the bovine brain and purified by the method of Yazawa *et al.* [15]. CaM was purified by precipitation with trichloroacetic acid, DEAE-cellulose chromatography and phenyl-Sepharose chromatography. The partially purified holoenzyme of cyclic AMP-dependent protein kinase II and adenylate cyclase was prepared from the human heart by the method of Beavo *et al.* [16] and Sulakhe *et al.* [17], respectively.

PDE activity was measured by the two-step assay, as described [19]. Unless otherwise noted, the enzymatic reaction was in a total volume of 0.5 ml containing buffer (50 mM Tris–HCl, pH 8.0 and 5 mM $MgCl_2$), substrate (0.4 μM cyclic [^{3}H]adenosine monophosphate or 0.4 μM cyclic [^{3}H]guanosine monophosphate), and enzyme preparation. The reaction was run at 30° for 15 min before termination by boiling for 5 min. 5′-[^{3}H]AMP or 5′-[^{3}H]GMP formed by the phosphodiesterase is converted to [^{3}H]adenosine or [^{3}H]guanosine by the action of nucleotidase and the product isolated by cation exchange resin was counted in a liquid scintillation counter. Cyclic AMP-dependent protein kinase activity was assayed by our method [19]. The activity of adenylate cyclase were determined by the method of Nakazawa *et al.* [20]. Cyclic-[^{3}H]AMP (24 Ci/mmol), cyclic-[^{3}H]GMP (15 Ci/mol), [^{3}H]ATP (25 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Amersham. Unlabeled cyclic AMP (cAMP), cyclic GMP (cGMP), snake venom (*Cro-*

† To whom all correspondence should be addressed.

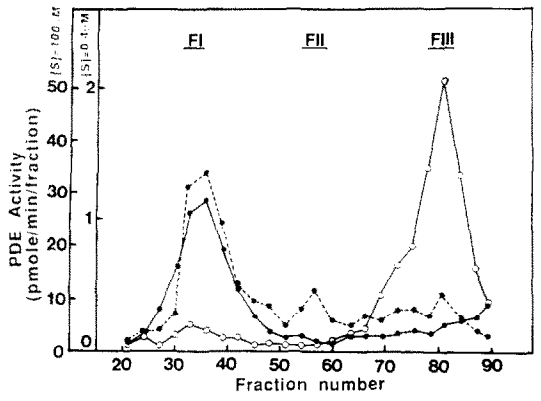

Fig. 1. DEAE-cellulose column elution profile of cyclic nucleotide phosphodiesterases from human heart. A linear gradient from 0 to 0.5 M sodium acetate was applied from fraction 20 to 90. Aliquots, 0.2 ml for high substrate (100 μM) or 0.1 ml for low substrate level (0.4 μM), were assayed directly as described under Materials and Methods: —○—, cyclic AMP (0.4 μM) hydrolysis; —●—, cyclic GMP (0.4 μM) hydrolysis; --●--, cyclic GMP (100 μM) hydrolysis.

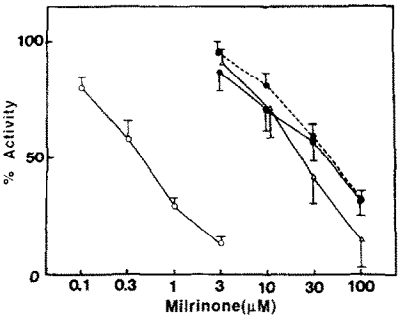

Fig. 2. Inhibition of human cardiac phosphodiesterases by milrinone. The activity of FI PDE was assayed as described under Materials and Methods with 0.4 μM [^{3}H]cGMP in the presence (--●--) or absence (—●—) of Ca^{2+}–CaM complex. The activity of FII (—△—) and FIII (—○—) PDE were determined in the presence of 1 mM EGTA with 0.4 μM [^{3}H]cGMP and 0.4 μM [^{3}H]cAMP, respectively. Activities are reported relative to that in the absence of milrinone. Points represent the mean ± SD of four experiments. Each experiment was run in duplicate.

talus atrox) were purchased from Sigma Chemical Co. (St Louis, MO). All other chemicals were of reagent grade or the best commercially available.

RESULTS

Findings in the case of DEAE-cellulose chromatographic separation of three forms of PDEs from human heart are shown in Fig. 1. These forms were designated FI, FII and FIII, according to the order of elution from the column. FI eluted at about 0.08 M sodium acetate, FII at about 0.2 M and FIII at about 0.35 M. FI PDE is stimulated by the Ca–CaM complex and has a higher affinity for cGMP. FII PDE showed a relative lower substrate affinity for both nucleotides, but hydrolyzed cGMP more rapidly than cAMP. FIII PDE has a low K_m (0.3 μM) for cAMP and was relatively specific for this substrate.

Figure 2 shows that milrinone inhibited human cardiac PDEs, to different degrees and the concentration required to produce 50% inhibition of the enzyme activity (IC_{50}) for FIII PDE was 0.42 μM. On the other hand, IC_{50} values for FI PDE in the presence or absence of Ca–CaM complex and FII PDE were 44, 38 and 19 μM, respectively. Milrinone proved to be a potent and selective inhibitor of human cardiac FIII PDE, cyclic AMP phosphodiesterase (cAMP-PDE). Table 1 summarizes these effects on human cardiac PDEs, as compared with reference drugs, papaverine and dipyridamole. Milrinone was about 50 times more potent than dipyridamole and more selective than papaverine in inhibiting human cardiac cAMP-PDE. Among the reference drugs investigated, papaverine potently inhibited cAMP-PDE (FIII), as it does human platelet cAMP-PDE [2]. Dipyridamole is a selective inhibitor of human platelet cGMP-PDE (FI) [21] and exhibited relatively little selectivity for any heart PDEs.

This inhibition of cAMP-PDE by milrinone was further analyzed by means of Lineweaver–Burk and Dixon plots. As shown in Fig. 3A, milrinone

Table 1. Effects of milrinone on human cardiac cyclic nucleotide phosphodiesterases

	IC_{50} (μM)*			
	FI†			
	EGTA	Ca^{2+}-CaM	FII‡	FIII§
Milrinone	38 ± 8	44 ± 7	10 ± 4	0.42 ± 0.10
Papaverine	8.7 ± 3.0	8.8 ± 1.0	15 ± 5	0.29 ± 0.04
Dipyridamole	29 ± 7	30 ± 4	18 ± 9	23 ± 3

* The IC_{50} value is defined as the concentration of drug required to produce 50% inhibition of enzyme activity. Each value is mean ± SD of four experiments.

† The activity of FI PDE was assayed with 0.4 μM [^{3}H]cGMP in the presence of 1 mM EGTA or Ca^{2+}–CaM complex.

‡ The activity of FII PDE was assayed with 0.4 μM [^{3}H]cGMP in the presence of 1 mM EGTA.

§ The activity of FIII PDE was assayed with 0.4 μM [^{3}H]cAMP in the presence of 1 mM EGTA.

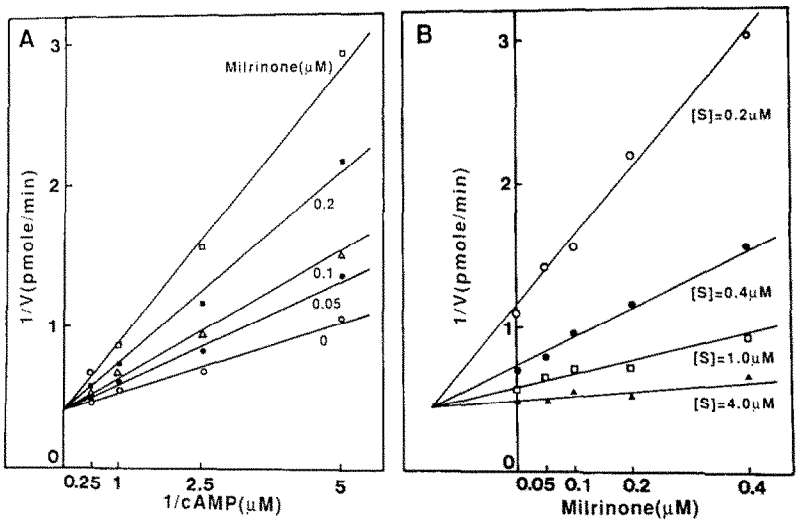

Fig. 3. Kinetic analysis of milrinone of human heart cyclic AMP phosphodiesterase (FIII) (A) Lineweaver–Burk plot; (B) Dixon plot; [S], substrate (cAMP) concentration. Each point represents the mean of duplicate assays.

inhibited cAMP-PDE, competitively with respect to cAMP. The Dixon plot (Fig. 3B) showed that the K_i value of milrinone for the enzyme was 0.15 μM. The inhibitory effects of milrinone on cAMP-PDE from various tissues, including platelets, aorta, kidney, lung and liver, were investigated, as compared with findings with either papaverine or dipyridamole. As shown in Table 2, the IC_{50} values of milrinone for heart, platelets and aorta cAMP-PDE were much the same. These values were about 28, 12 and 7 times less than that for kidney, lung and liver enzyme, respectively. Milrinone inhibited more selectively cAMP-PDEs from cardiovascular associated tissues than from others. On the other hand, cAMP-PDEs from heart, platelet and aorta were inhibited less effectively by dipyridamole than those from other tissues. There was little difference in the effects of papaverine for cAMP-PDEs from various tissues.

We also investigated the effects of milrinone on other cAMP-associated enzymes, cAMP-dependent protein kinase II (PK-A) and adenylate cyclase from the human heart. Milrinone, up to 100 μM, neither inhibited the cAMP-induced PK-A activity nor activated the PK-A instead of cAMP (data not shown). However, milrinone slightly inhibited the cAMP-induced stimulation of PK-A with 10 μM ATP, at a concentration of over 300 μM. Milrinone also did not alter the affinity of cAMP for the activation of PK-A. Milrinone, up to 1 mM, had no effects on the activity of human cardiac adenylate cyclase, in the absence or presence of NaF and norepinephrine (data not shown). The affinity of norepinephrine for the activation of adenylate cyclase was not affected by milrinone (300 μM).

DISCUSSION

Milrinone was shown to be a potent and specific inhibitor of human cardiac cAMP-PDE (FIII PDE), a "low K_m" cAMP phosphodiesterase. Although human cardiac FI and FII PDE were also inhibited by milrinone, this drug had at least a 50–100-fold greater affinity for the cAMP-PDE (FIII-PDE). These inhibitory effects of milrinone specifically for cAMP-PDE were also observed in guinea-pig [1], rat [2] and bovine hearts [3]. Bipyridine derivatives such as milrinone and amrinone have potent inotropic effects and vasodilatory properties [1, 22] with increased intracellular cyclic AMP levels [23]. As the concentration of milrinone which inhibited the activity of PDEs had no significant effects on cAMP-dependent protein kinase or on adenylate cyclase from the human heart, the positive inotropic

Table 2. Effects of milrinone on cyclic AMP phosphodiesterases from various human tissues

	IC_{50} (μM)*					
	Heart	Platelet	Aorta	Kidney	Lung	Liver
Milrinone	0.42 ± 0.10	0.42 ± 0.05	0.42 ± 0.08	12 ± 4	5.2 ± 2.0	2.9 ± 0.6
Papaverine	0.29 ± 0.04	0.38 ± 0.04	0.33 ± 0.05	0.56 ± 0.15	0.53 ± 0.08	0.47 ± 0.05
Dipyridamole	23 ± 3	19 ± 3	15 ± 2	3.9 ± 0.8	2.1 ± 0.3	2.9 ± 0.3

* The IC_{50} value is defined as the concentration eliciting 50% inhibition of cAMP phosphodiesterase. The enzymes from various human tissues were assayed under Materials and Methods with 0.4 μM [^{3}H]cAMP in the presence of 1 mM EGTA. Each value is mean ± SD of four experiments.

effect of this drug for the human heart may be mainly due to the inhibition of cAMP-PDE. However, other types of action besides the inhibitory effect of cAMP-PDE have been reported [1].

With regard to the potential differences of milrinone in inhibiting cAMP-PDEs from various tissues, milrinone is the more potent inhibitor of cAMP-PDEs in human heart, platelet and aorta, as compared to findings in other tissues including the kidney, liver and lungs. It is of interest that dipyridamole is potent in inhibition of FIII PDEs from human kidney, lung and liver, and is less potent on those from human cardiac muscle, aorta and platelets. However, papaverine, which is also a potent and a selective inhibitor of cAMP-PDE, showed no variations in sensitivity of the different cAMP-PDEs from various human tissues. These differences in the selectivity of milrinone were evident also in the case of OPC-3689, cilostamide, in various human tissues [23]. Milrinone and cilostamide are two cAMP-PDE inhibitors that are structurally dissimilar but which possess a similar tendency in inhibiting human cAMP-PDEs. The kinetic analysis of the competitive inhibition of cAMP hydrolysis by milrinone suggests that this drug seems to bind the region of the catalytic site of the enzyme. Cyclic AMP-PDEs in all tissues except aorta revealed normal kinetics for cAMP hydrolysis with K_m values ranging from 0.30 to 1.0 μM [12–14]. Therefore, the difference of sensitivity of milrinone in inhibiting cAMP-PDEs is not due to differences in the K_m values of the enzyme for the substrate. These results suggest that milrinone as well as cilostamide may recognize the different forms of human cAMP-PDEs. The "low K_m" cAMP phosphodiesterase (cAMP-PDE) has been purified from various mammalian tissues, including human platelets [6, 7], human lung [8], dog kidney [9] and bovine heart [3]. There was no uniformity and the size, affinity for substrates and catalytic rate differed considerably. Yamamoto *et al.* reported that there were two distinct low K_m cAMP-PDE in calf liver and that they differed from cilostamide and RO 20-1724 in their inhibitory effects as well as in size and substrate specificities [10]. Harrison *et al.* demonstrated that antiserum directed against the bovine cardiac cGMP-inhibited phosphodiesterase did not cross-react with bovine lung "low K_m" phosphodiesterase [11]. All these observations suggest that there are different forms of cAMP-PDEs in various tissues of various species.

Milrinone may be effective not only for treating heart failure but also for elucidating the molecular differences in cAMP-PDEs from various human tissues.

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