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Milrinone and thyroid hormone stimulate myocardial membrane Ca^{2+}-ATPase activity and share structural homologies

(thyromimetic activity/molecular conformation/inotropic agent/sarcolemma/calcium pump)

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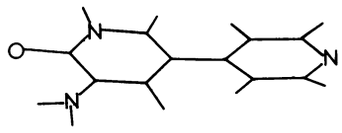
ABSTRACT **We have recently shown that thyroid hormone in physiological concentrations stimulates sarcolemma-enriched rabbit-myocardial-membrane Ca^{2+}-ATPase *in vitro*. In this study, milrinone [2-methyl-5-cyano-(3,4′-bipyridin)-6(1H)-one], a cardiac inotropic agent, was thyromimetic in the same system. At clinically achievable concentrations (50–500 nM), milrinone significantly stimulated membrane Ca^{2+}-ATPase *in vitro*. This action was antagonized by W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide], an agent that also blocks thyroid hormone action on the Ca^{2+}-ATPase, at concentrations as low as 5 μM. Progressive additions of milrinone to membranes incubated with a fixed concentration of thyroxine (0.10 nM) or triiodothyronine resulted in a progressive obliteration of the thyroid hormone effect on Ca^{2+}-ATPase. Amrinone [5-amino-(3,4′-bipyridin)-6(1H)-one], the parent bipyridine of milrinone, had no effect on myocardial Ca^{2+}-ATPase activity. X-ray crystallographic analysis of milrinone and amrinone revealed structural homologies between the phenolic ring of thyroxine and the substituted ring of milrinone, whereas amrinone did not share these homologies. The mechanism(s) of the inotropic actions of thyroxine and of milrinone is not clearly understood, but these observations implicate Ca^{2+}-ATPase, a calcium pump-associated enzyme, as one mediator of the effects on the heart of these two compounds.**

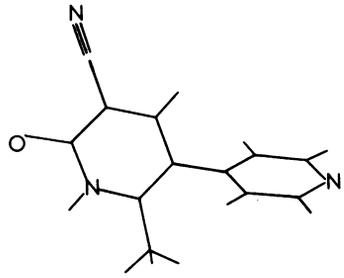
Milrinone [2-methyl-5-cyano-(3,4′-bipyridin)-6(1H)-one] is a nonglycosidic positive-inotropic bipyridine (1, 2) whose biochemical mechanism of action on the heart has not been established. The action of the bipyridines could involve either phosphodiesterase inhibition and cyclic AMP accumulation (3–5) or increasing the rate of calcium sequestration (6). The drug has a bipyridine structure whose substituted ring has features that might mimic those of the thyroid hormones, thyroxine (T_4) or triiodothyronine (T_3); this caused us to examine milrinone for stimulatory action on rabbit myocardial membrane Ca^{2+}-ATPase activity. We have previously reported that physiological concentrations of thyroxine (0.10 nM) stimulate this myocardial membrane calcium pump-associated enzyme *in vitro* via a calmodulin-dependent process (7). This Ca^{2+}-ATPase is thought to be responsible for calcium ejection by myocardial cells during rest.

MATERIALS AND METHODS

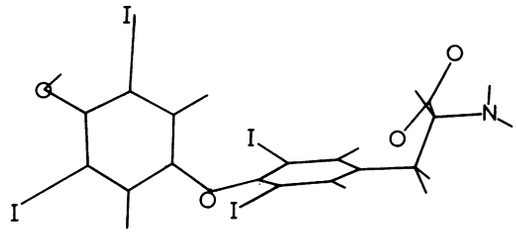
Reagents and Hormones. Na_2ATP, sodium orthovanadate, L-thyroxine (T_4), and 3,5,3′-triiodo-L-thyronine (T_3) were obtained from Sigma. [^{125}I]T_4 was purchased from Amersham and its purity verified by thin layer chromatography (8). ^{45}Ca was obtained from ICN Radioisotopes (Cambridge, MA).



Amrinone



Milrinone



Thyroxine

Milrinone and amrinone were kindly supplied by Sterling-Winthrop Research Laboratory (Rensselaer, NY). W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide] was obtained from CAABCO (Houston, TX).

Rabbit Myocardial and Erythrocyte Membranes. Rabbit myocardial membranes enriched in sarcolemma were prepared by the method of Jones *et al.* (9) from mature New Zealand White does and bucks. Electron microscopic analysis of the preparations revealed 70% of the preparation consisted of vesicles with no myofibrillar elements. These membranes exhibit up to 26-fold increase in 5′-nucleotidase activity, when compared to crude membranes, and contain a Ca^{2+}-ATPase that was sensitive to inhibition with 2 μM sodium orthovanadate (7), consistent with sarcolemma enrichment. Rabbit erythrocyte membranes were prepared from hypotonically lysed erythrocytes, as described (10).

Ca^{2+}-ATPase Assay. Enzyme activity was determined as reported (7), with an assay standardized in rabbit myocardial and erythrocyte membranes. Activity was defined as the

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Abbreviations: T_4, L-thyroxine; T_3, 3,5,3′-triiodo-L-thyronine; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

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difference in ATP hydrolysis in the presence and absence of 20 μM Ca^{2+} (7) and expressed as μmol of P_i liberated per mg of membrane protein per 90 min. Protein was determined by the Lowry method (11), with bovine serum albumin as a standard. The results represent means of three or more experiments performed in duplicate.

Effects of Thyroid Hormone and Milrinone *in Vitro* on Ca^{2+}-ATPase. Milrinone, without or with thyroid hormone (T_4 or T_3), was added in varying concentrations to membrane preparations and incubated for 90 min at 37°C in the course of the enzyme assay. Control incubations included the diluent for milrinone or T_4 without active substance. In studies involving an inhibitor of calmodulin actions, W-7 (12) was added in varying concentrations to aliquots of myocardial membranes and was incubated with milrinone or T_4. In selected studies, amrinone, the parent compound of milrinone, was added to membranes (without and with T_4) to determine the bioactivity of this bipyridine on Ca^{2+}-ATPase.

Uptake of ^{45}Ca by Myocardial Membranes. To assess the possibility that milrinone acted as an ionophore on cardiac membranes, ^{45}Ca, as tracer in 1 mM unlabeled Ca^{2+}, was added to membranes in the presence of W-7 (100 μM) or sodium orthovanadate (50 μM), which inhibit the calcium pump activity. Following incubation at 20°C for 60 min, the vesicles were washed and pelleted by centrifugation. The amount of radioactivity associated with the vesicles was expressed as percent of ^{45}Ca added to the incubation mixture.

Statistical Analysis. The statistical significance of differences in enzyme activity in the presence and absence of milrinone and of thyroid hormone was estimated by two-way analysis of variance and paired *t* test.

Structural Analysis. Crystals of milrinone and amrinone were grown at room temperature from ethanol solutions. A rectangular plate-shaped crystal (0.16 mm × 0.32 mm × 0.54 mm) of milrinone was used to measure the crystal data: $C_{12}H_9ON_3$; M_r = 210.2; space group $P2_1/c$, a = 7.067(1), b = 10.089(1), c = 15.477(2) Å, β = 100.74(1)°, volume = 1082.2 Å^3, z = 4. The intensities of diffracted x-rays were measured on an Enraf-Nonius (Bohemia, NY) CAD-4 automated diffractometer using copper radiation by the $\Theta - 2\Theta$ scan technique; 2248 independent data points were collected of which 1995 were considered observed, $I > 2\sigma(I)$. The structure was solved by using direct methods programs MULTAN (13) and NQEST (14). All nonhydrogen atoms were located in the first electron-density map and the hydrogen atoms were located in subsequent difference Fourier synthesis maps. The nonhydrogen atoms were refined anisotropically and the hydrogen atoms were refined isotropically. Full matrix least-squares refinement was carried out and the residual, $R = \Sigma(|F_o| - |F_c|)/\Sigma|F_o|$, is 0.08 for all data.

Crystal data for a plate-shaped crystal (0.08 mm × 0.28 mm × 0.40 mm) of amrinone were as follows: $C_{10}H_9ON_3$; M_r = 187.2; space group $P2_1/c$, a = 9.257(5), b = 17.064(6), c = 22.844(6) Å, β = 99.70(5)°, volume = 3557.2(6) Å^3, z = 16. X-ray diffraction intensities were collected on a Nicolet (Cupertino, CA) P3 automated diffractometer using molybdenum radiation by the $\Theta - 2\Theta$ scan technique; 6274 independent data points were collected of which 4193 were considered observed, $I > 2\sigma(I)$. The structure was solved using direct methods techniques. All nonhydrogen atoms were located in the first electron density map. Nonhydrogen atoms were refined anisotropically, and the hydrogen atoms were held fixed in their calculated positions. Full-matrix least-squares refinement was carried out, and the residual was 0.09 for all data.

RESULTS

Action of Milrinone and Amrinone on Ca^{2+}-ATPase *in Vitro*. Milrinone was found to be a stimulator of myocardial membrane Ca^{2+}-ATPase *in vitro* (Fig. 1). The concentration dependence of the milrinone effect included maximum activity at 100 nM (73% increase in enzyme activity compared to control samples lacking milrinone, $P < 0.001$). In various cardiac membrane preparations, the increase in enzyme activity *in vitro* attributable to milrinone was 25–100%. The maximum effective concentration of milrinone (100 nM) is clinically achievable (1). The dose–response curve was parabolic, with a decreasing effect of milrinone seen at 500 and 1000 nM. A parabolic dose–response curve is characteristic of other compounds known to stimulate Ca^{2+}-ATPase activity, including low concentrations of flavonoids (15) and thyroid hormones (16, 17). Milrinone stimulation of the enzyme was unchanged by the omission of sodium from the incubation buffer.

Amrinone, the parent bipyridine of milrinone, did not affect the basal activity of myocardial Ca^{2+}-ATPase and did not inhibit the *in vitro* action of thyroid hormone on the enzyme (Fig. 2).

We have shown similarities between the Ca^{2+}-ATPases of myocardial membranes (7) and of the erythrocyte membrane (10, 16, 17) with respect to stimulation *in vitro* by iodothyronines. Milrinone (10–1000 nM) had no effect on either basal or T_4-stimulated rabbit erythrocyte membrane Ca^{2+}-ATPase activity (results not shown).

Action of Thyroid Hormone on Myocardial Ca^{2+}-ATPase Activity in the Presence of Milrinone. The stimulatory effect of T_4 (0.10 nM) *in vitro* on myocardial membrane Ca^{2+}-ATPase activity in the absence and presence of various concentrations of milrinone is shown in Fig. 3. Increasing the concentration of milrinone reduced the effect of the iodothyronine on Ca^{2+}-ATPase until, at 1 μM milrinone, no hormonal action was observed. Similarly, additions of milrinone progressively inhibited the stimulatory effect of T_3 (0.10 nM) on Ca^{2+}-ATPase activity (data not shown).

Myocardial Ca^{2+}-ATPase Activity *in Vitro* in the Presence of W-7. W-7, a calmodulin inhibitor, decreased basal Ca^{2+}-ATPase activity and the stimulatory effect of milrinone (100 nM) on Ca^{2+}-ATPase (Fig. 4). The W-7 effect was concentration dependent. W-7 has previously been shown to inhibit T_4 stimulation of myocardial membrane Ca^{2+}-ATPase activity *in vitro* (7) and to inhibit the effect of T_4 on Ca^{2+}-ATPase in the presence of milrinone (Fig. 4). There is a parallel effect

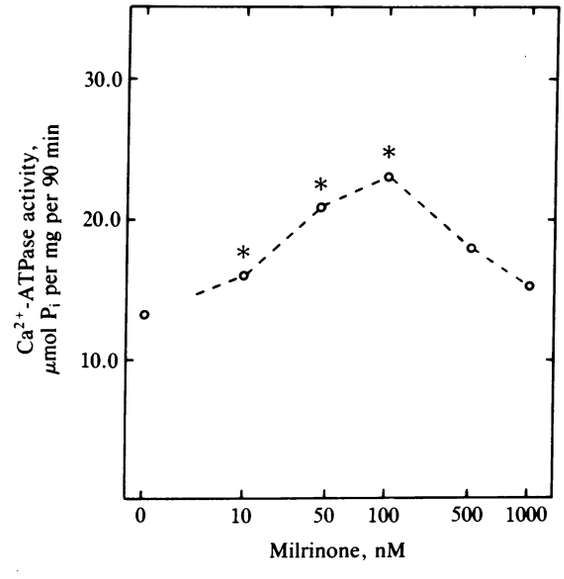

FIG. 1. Stimulation of rabbit myocardial membrane Ca^{2+}-ATPase activity with increasing concentrations of milrinone. *, Significant ($P < 0.01$) stimulation of enzyme activity.

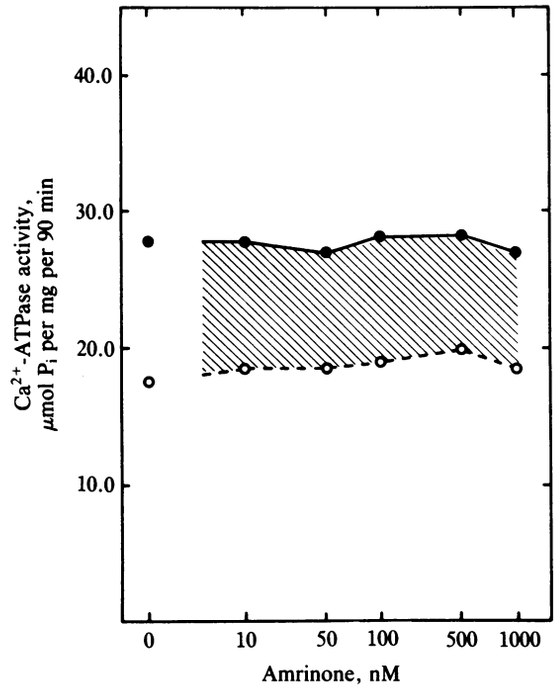

FIG. 2. Lack of effect of amrinone on the basal activity of rabbit myocardial membrane Ca^{2+}-ATPase (open circles) and thyroxine stimulation of enzyme activity (solid circles).

of the action of W-7 on milrinone and T_4. Parallel inhibition by W-7 of thyroid hormone and milrinone action is consistent with a common mechanism of action of these two compounds.

Lack of Ionophore Effect of Milrinone on Calcium Uptake by Myocardial Membranes *in Vitro*. Since milrinone could have calcium ionophore properties that secondarily result in increased Ca^{2+}-ATPase activity in membrane vesicles, the myocardial membrane uptake of ^{45}Ca in the presence of W-7 or vanadate (to inhibit the calcium pump) was investigated.

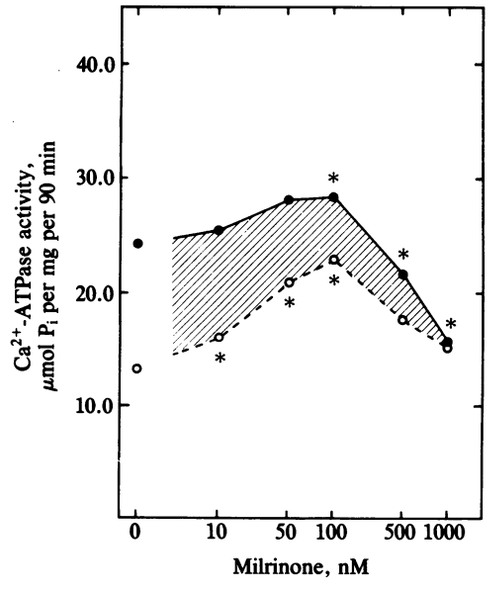

FIG. 3. Stimulation of rabbit myocardial membrane Ca^{2+}-ATPase activity with increasing concentrations of milrinone (open circles) and with a constant amount of thyroxine (0.10 nM) (solid circles). *, Significant ($P < 0.01$) stimulation of basal activity or inhibition of hormone effect.

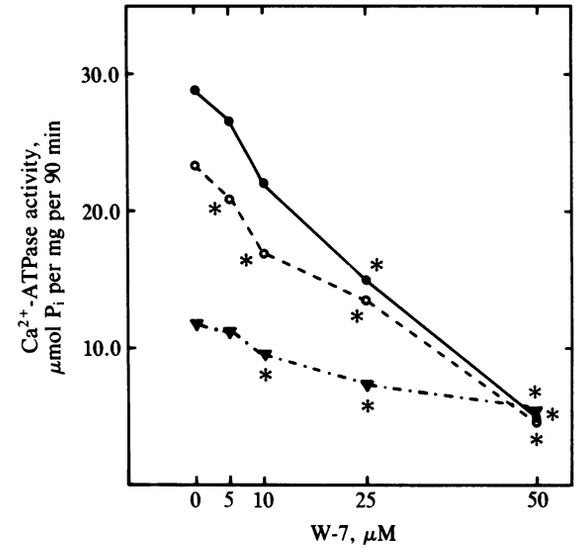

FIG. 4. Inhibitory effect of increasing concentrations of W-7 on basal Ca^{2+}-ATPase activity (triangles), stimulation of Ca^{2+}-ATPase activity by milrinone (open circles), and stimulation of Ca^{2+}-ATPase activity by milrinone and thyroxine (solid circles). *, Significant ($P < 0.02$) inhibition of basal activity or inhibition of enzyme stimulation by milrinone or thyroxine.

The calcium ionophore, A23187 (10 μM), significantly increased vesicle-associated ^{45}Ca [0.69%, control vs. 1.24%, A23187 ($P < 0.01$) in the presence of W-7; 1.03%, control vs. 2.58%, A23187 ($P < 0.01$) in the presence of vanadate]. In contrast, milrinone (10 or 100 nM) did not increase vesicle ^{45}Ca above control in the presence of either W-7 or vanadate. Thyroid hormone did not act as an ionophore in this system (data not shown).

Structural Results. The molecular conformations of amrinone and milrinone are shown in Fig. 5. The four independent amrinone molecules in the unit cell of the crystal are nearly coplanar with the two pyridine rings twisted 11°,

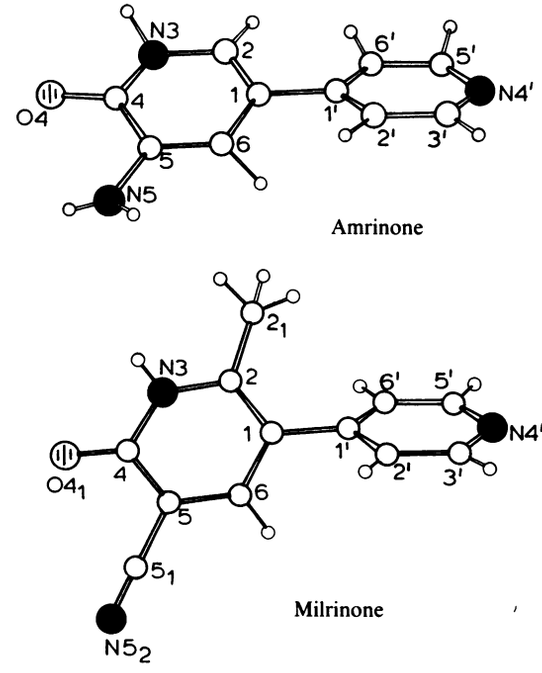

FIG. 5. Molecular conformation and numbering scheme of amrinone (molecule 3 in the asymmetric unit) and milrinone.

10°, 9°, and 24° from one another for molecules 1 to 4, respectively. The angle between the two pyridine rings is 44° in milrinone as a result of the steric bulk of the 2-methyl substituent.

To understand the thyromimetic potential of milrinone in this enzyme system, the molecular structure of milrinone was compared with that observed for thyroxine (18). Crystal structure analysis of thyroxine shows that the diphenyl ether conformation is twist-skewed; that is, the two iodophenyl rings are nearly perpendicular and bisecting with respect to one another. The torsion angles about the ether bond are −113° and 33° for the tyrosyl and phenolic bonds, respectively. Conformational analysis of these two structures using computer graphic techniques shows (Fig. 6) that the best fit of the two structures results from the superposition of the milrinone substituted ring with the iodophenolic ring of T_4 (Fig. 6*a*). In this orientation the 5-cyano group fits into the space occupied by the 5′-iodine of T_4 and the 4-keto group occupies the same space as the 4′-hydroxyl group of the hormone. This superposition provides the best structural homology between the chemical and electronic properties of the hormone since a similar fit of milrinone's substituted ring on the tyrosyl ring of thyroxine will not have the same electronic and hydrogen bonding potential as the 4′-hydroxyl

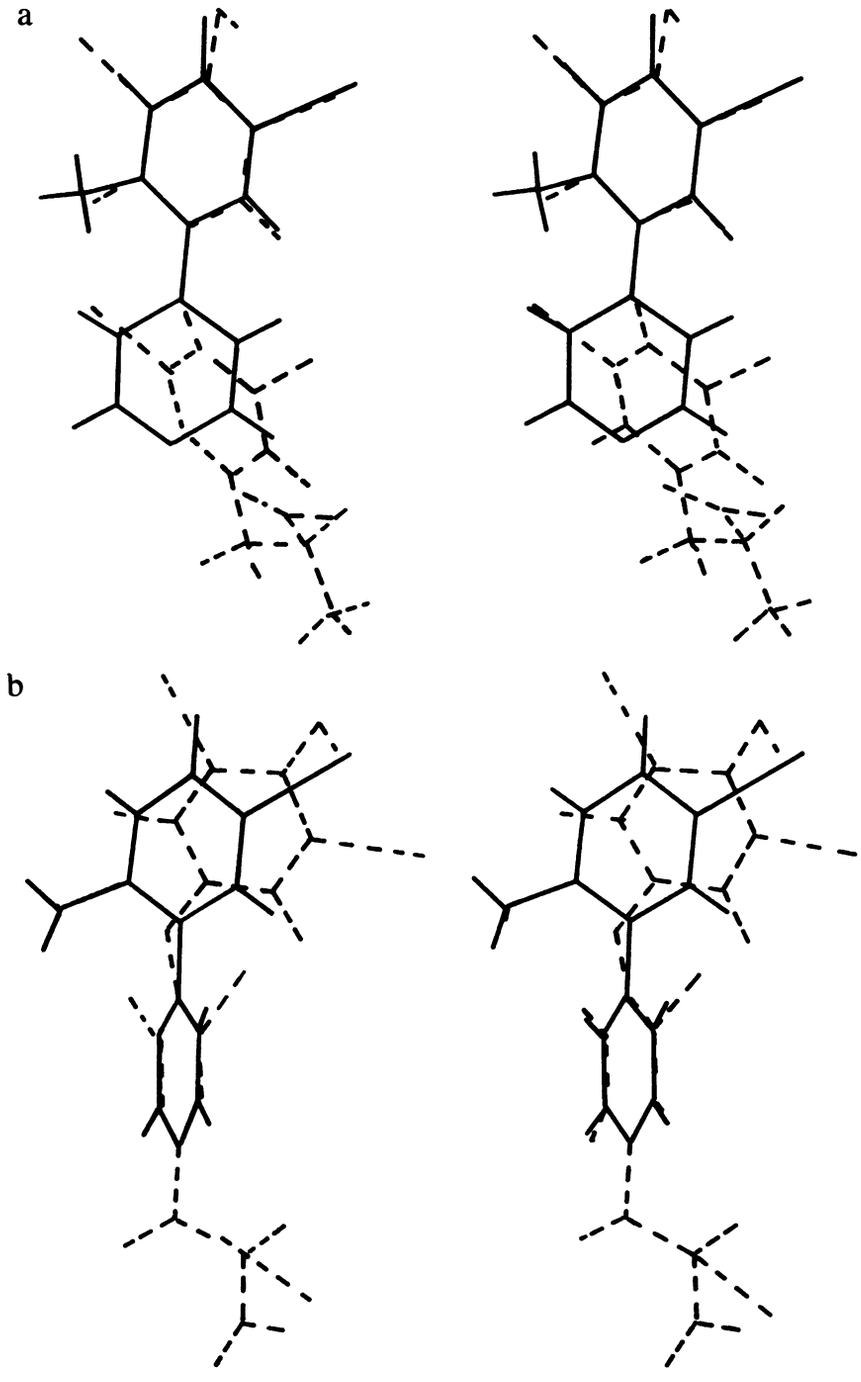

FIG. 6. (*a*) Stereo drawing showing the superposition of the substituted pyridine ring of milrinone (solid lines) on the phenolic ring of thyroxine (dashed lines). Note that the cyano group occupies the same space as the 5′-iodo group and that the keto and phenolic oxygens overlap in this orientation. (*b*) Stereo drawing showing the superposition of the unsubstituted pyridine ring of milrinone (solid lines) on the tyrosyl ring of thyroxine (dashed lines).

group. There is no 2'-methyl substituent in thyroxine. On the other hand, if the superposition is made with the tyrosyl ring of T_4 and the substituted milrinone ring, there is not much volume shared in common (Fig. 6*b*). There is even less homology between the structure of amrinone and T_4. In this case, the amrinone 5-amino does not occupy the same volume as the cyano group, nor does it have the same electrochemical potential.

DISCUSSION

In this study, milrinone, a bipyridine compound with inotropic cardiac effects (1), has been shown for the first time to be thyromimetic in an *in vitro* myocardial membrane Ca^{2+}-ATPase model system. This sarcolemma-enriched preparation has been shown to respond to subnanomolar concentrations of thyroid hormone in a calmodulin-dependent manner (7). Thyroid hormone is known to have chronotropic and inotropic effects on the heart (19) and, in human studies, milrinone has been found to increase heart rate as well as to increase myocardial contractility (1). The inotropic activity of iodothyronines has been attributed, at least in part, to stimulation of myosin-associated Ca^{2+}-ATPase (20). The inotropism of the bipyridines has been thought to depend upon the inhibition of phosphodiesterases (3–5), in contrast to earlier studies (21). Changes in the rate of intracellular calcium sequestration have also been implicated (6).

Phosphodiesterase antagonism has been studied only in the case of the parent bipyridine, amrinone, which had an IC_{50} of 40 μM in dog myocardium (3). In the present studies, stimulation of rabbit myocardial membrane Ca^{2+}-ATPase activity by milrinone was maximal at 0.1 μM and was not achieved at any concentration of amrinone. In intact animals, the direct inotropic effect of amrinone may, in fact, be less important than its effect on afterload reduction (22). In contrast, the action of milrinone on cardiac output is expressed through enhanced myocardial contractility (1). Clinical effects of amrinone and milrinone, therefore, may depend upon more than one biochemical mechanism.

Progressive diminution of thyroid hormone effect on myocardial membrane Ca^{2+}-ATPase activity with additions of milrinone is consistent with a shared biochemical mechanism of action at the enzyme. Amrinone stimulates neither membrane Ca^{2+}-ATPase nor heart rate (23). Such observations suggest that sarcolemmal Ca^{2+}-ATPase participates in the chronotropic actions of milrinone and thyroid hormone, although other mechanisms (such as heightened adrenergic responsiveness in the presence of iodothyronines) are possible. If the myocardial sarcoplasmic reticulum (SR) Ca^{2+}-ATPase resembles the enzyme in sarcolemma in terms of susceptibility to stimulation by thyroid hormone, then stimulation of the SR enzyme by both milrinone and T_4 could be inotropic (increasing SR Ca^{2+} stores prior to contraction) and also speed relaxation (rate of uptake of Ca^{2+} by SR). Preliminary studies of T_4 and milrinone in an SR-enriched membrane fraction from rabbit heart, prepared in our laboratory by Percoll gradient centrifugation, indicates that the Ca^{2+}-ATPase of these membranes is also stimulated *in vitro* by thyroid hormone and milrinone (unpublished results).

While the Ca^{2+}-ATPase of sarcolemma and the erythrocyte membrane are remarkably similar enzymes (24), the cell membranes are very different. Hence, it is not surprising that milrinone acts only on sarcolemma, whereas thyroid hormone, which has a diiodotyrosyl ring, acts on both membranes. We have elsewhere emphasized the importance of both the tyrosyl and phenolic rings of the iodothyronines in their stimulation of erythrocyte Ca^{2+}-ATPase (25).

The crystallographic studies reported here indicate that the best structural homologies between T_4 and milrinone are with the phenolic ring of T_4 and the substituted ring of milrinone. Sterically, the 5-cyano group of milrinone occupies the same volume as that of the 5'-iodine, and the 4'-oxygen functions overlap. This study further suggests that it is the steric or electronic effect of the cyano group of milrinone that is important for the observed Ca^{2+}-ATPase activity since amrinone, which has an amino group instead of cyano, is inactive. The presence of the 2-methyl in milrinone may enhance its activity by forcing the two pyridine rings to adopt a more twisted conformation (e.g., 44° vs. 10°). Thus, it is of interest to test other inotropic bipyridine analogues in this enzyme system.

Results of electrophoretic studies in our laboratory indicate that milrinone (1–10 μM) competes with T_4 for binding sites on human serum prealbumin (unpublished results). Amrinone has less than 10% of the competitive activity of milrinone. Thus, expression of the structural homologies of milrinone and iodothyronine is not limited to the myocardial membrane calcium pump.

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