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CALCIUM CHANNEL BLOCKER INHIBITION OF THE CALMODULIN-DEPENDENT EFFECTS OF THYROID HORMONE AND MILRINONE ON RABBIT MYOCARDIAL MEMBRANE Ca^{2+}-ATPase ACTIVITY*

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Abstract—The Ca^{2+}-ATPase activity of rabbit myocardial membranes is stimulated *in vitro* by L-thyroxine and by milrinone, a bipyridine. These effects are concentration dependent and calmodulin requiring. The calcium channel blockers nifedipine and verapamil have been reported to have anti-calmodulin effects in other assay systems. In this study we have examined the effects of nifedipine and verapamil on rabbit myocardial membrane Ca^{2+}-ATPase activity, in the absence (basal activity) and presence of exogenous L-thyroxine (T_4), 10^{-10} M, and milrinone, 10^{-7} M. Basal enzyme activity was inhibited by a minimum of 10^{-6} M nifedipine (IC_{50} of 3.4×10^{-5} M) and 10^{-5} M verapamil (IC_{50} of 1.5×10^{-4} M). Both calcium antagonists inhibited enzyme stimulation by T_4 and milrinone, with half-maximal inhibition of T_4 and milrinone effects, respectively, at 2.9×10^{-5} M and 9.0×10^{-6} M nifedipine and 3.0×10^{-5} M and 5.2×10^{-5} M verapamil. The addition of exogenous purified calmodulin, 40 ng/μg membrane protein, in the presence of 10^{-5} M nifedipine or verapamil restored T_4-stimulated enzyme activity. Nifedipine and verapamil, each at a concentration of 10^{-6} M, significantly inhibited binding of radioiodinated calmodulin to rabbit heart membranes *in vitro*. These studies provide evidence that nifedipine and verapamil have an anti-calmodulin effect in this myocardial enzyme system. Through interaction with calmodulin, the channel blockers inhibit thyroid hormone and milrinone stimulation of myocardial membrane Ca^{2+}-ATPase.

The Ca^{2+}-stimulable, Mg^{2+}-dependent adenosine triphosphatase (Ca^{2+}-ATPase) activity of rabbit myocardial membranes is stimulated *in vitro* by physiologic concentrations of L-thyroxine (T_4) and 3,5,3′-triiodo-L-thyronine (T_3) [1]. This extranuclear membrane action of thyroid hormone is dose-dependent, with a maximal effect seen at 10^{-10} M, and is inhibited by trifluoperazine and *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7), two pharmacologic inhibitors of calmodulin action [1, 2]. The effect of T_4 in this enzyme system is reproduced by milrinone [3], a bipyridine compound which shares structural homologies with thyroid hormone and is a positive cardiac inotropic agent [4]. The positive inotropic effect of milrinone may be due, in part, to stimulation of sarcoplasmic reticulum Ca^{2+}-ATPase [3] in addition to its known inhibitory effect on phosphodiesterase [5–7] and its effect on the rate of intracellular calcium sequestration [8, 9]. The milrinone effect on Ca^{2+}-ATPase is dose-dependent, with maximal stimulation at a concentration of 10^{-7} M. Enzyme stimulation by milrinone is blocked by W-7, suggesting, too, that calmodulin is required for the milrinone effect to be manifest.

We have demonstrated previously calmodulin-dependent T_4 stimulation of Ca^{2+}-ATPase activity in human erythrocyte membranes, employing an assay system similar to that for the myocardial membrane enzyme [2, 10]. The *in vitro* addition of the calcium channel antagonists nifedipine and verapamil (10–300 μM) to this red cell enzyme preparation causes inhibition of the T_4 effect on the enzyme [11]. It has been reported that calcium channel blockers may have an anti-calmodulin effect [12, 13] in addition to their effect on voltage-dependent calcium channels. In this study, we have employed the myocardial membrane Ca^{2+}-ATPase assay system to study the effects of verapamil and nifedipine on calmodulin-dependent T_4 and milrinone stimulation of the enzyme. Further, we have examined the interaction of channel blockers and exogenous calmodulin in this enzyme system and have studied the effects of nifedipine and verapamil on the binding of radioiodinated calmodulin to rabbit heart membranes.

METHODS

Reagents and hormones. Na_2ATP, L-thyroxine (T_4) and W-7 were obtained from Sigma (St. Louis, MO), and purified calmodulin was either of rat testis origin (CAABCO, Houston, TX) or prepared from beef brain in our laboratory by phenothiazine affinity chromatography [14]. ^{125}I for iodination of purified calmodulin was obtained from Amersham (Arlington Heights, IL). Nifedipine was provided by Pfizer, Inc. (Brooklyn, NY), verapamil by G. D. Searle &

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Co. (Skokie, IL), and milrinone by Sterling-Winthrop Research Institute (Rensselaer, NY).

Myocardial membrane preparation. Myocardial membranes were prepared by our modification [1] of the method of Jones *et al.* [15]. Following KCl/histidine extraction and differential centrifugation of rabbit ventricular homogenate, final membrane preparations were obtained. Earlier studies comparing 5′-nucleotidase activity in final membrane preparations with that in initial ventricular homogenate demonstrated a mean 20-fold (range 9–28, N = 4) increase in activity; 60% of the Ca^{2+}-ATPase activity of these preparations was also inhibited by 2 μM vanadate [1]. These findings are consistent with sarcolemmal enrichment of the membranes [16].

Previous studies demonstrated, by electron microscopy, a final membrane preparation of 70% vesicles, 30% membrane fragments, and no myofibrillar contamination [1]. Vesicles obtained with this technique have a primarily right side out orientation [15]. Latent membrane Na^+, K^+-ATPase activity was not present in our preparation (no increase in enzyme activity after exposure of membranes to sodium dodecyl sulfate [0.3 mg/ml]; F. B. Davis, P. J. Davis and S. D. Blas, unpublished observations), indicating that the ATPase activities and calmodulin response studies described below reflect the presence of membrane fragments and leaky vesicles.

Ca^{2+}-ATPase assay. Ca^{2+}-ATPase activity was assayed by our previously published method [1, 10] in which enzyme activity is specifically defined as the difference in hydrolysis of ATP in the presence and absence of Ca^{2+}. The reaction mixture contained 10 mM Tris (pH 7.4), 0.1 mM ethyleneglycolbis(amino-ethylether)tetra-acetate (EGTA), 1 mM $MgCl_2$, 75 mM NaCl, 25 mM KCl, 1 mM ATP, and 5 μg membrane protein per 2.5 ml of assay medium. Reactions were carried out at 37° in a covered incubator precluding light exposure. Selected samples contained 0.15 mM $CaCl_2$, resulting in an optimal free Ca^{2+} concentration of 20 μM as determined by ion-specific electrode and computer-assisted calculation [17]. Enzyme activity was expressed as μmoles inorganic phosphate (P_i) [18] liberated per mg membrane protein [19] per 60-min assay period.

The effects of thyroid hormone, milrinone, verapamil, nifedipine and calmodulin were determined by measurement of the difference between enzyme activities in the presence and absence of these factors; control membrane samples contained appropriate ligand diluent [10 mM Tris or 1% dimethyl sulfoxide (DMSO)]. All experiments were performed in duplicate on membranes from a single preparation. The results presented are means ± SE of three or more experiments. Statistical analysis was carried out by analysis of variance and Student's paired *t*-test. Pairing data for statistical analysis normalizes enzymatic data to its own control which adjusts for the variations in basal activity seen between different membrane preparations.

Calmodulin binding to myocardial membranes. Beef brain and rat testis calmodulins were radioiodinated according to the method of Graf *et al.* [20], and purified by Sephadex G-50 chromatography. Because of their immunologic identity [21], the two calmodulins were used interchangeably in binding studies. Tracer level [^{125}I]calmodulin was preincubated in 10 mM Tris buffer, pH 7.45, at 37° with nifedipine, verapamil, W-7 or control diluent (1% DMSO) for 15 min, after which rabbit myocardial membranes, 250 μg protein per 2 ml volume, were added and incubated for 1 hr. The incubation was terminated by ice immersion and centrifugation of membranes at 20,000 *g*, 4° for 20 min. The pellet was washed with 10 mM Tris and calmodulin binding to membranes was expressed as percent of total radioactivity added to each tube which was found in the pellet. Experimental samples were paired with their respective controls—which contained diluent for channel blockers, but no active drugs—for statistical analysis by Student's paired *t*-test.

RESULTS

Effects of nifedipine and verapamil on basal, T_4- and milrinone-stimulated Ca^{2+}-ATPase activity. Myocardial membrane Ca^{2+}-ATPase activity in the absence of T_4 and milrinone (basal activity) was 35.5 μmoles P_i per mg membrane protein per 60 min (Fig. 1). T_4, 10^{-10} M, increased enzyme activity by 32% ($P < 0.001$) and milrinone, 10^{-7} M, caused a 37% increase in enzyme activity ($P < 0.001$). These concentrations of T_4 and milrinone are optimal for stimulation of this enzyme in our assay system [1, 3].

Nifedipine, in concentrations of 10^{-6} to 10^{-3} M, inhibited basal myocardial membrane Ca^{2+}-ATPase activity in a dose-dependent manner ($P < 0.001$, Fig. 2A). The minimum concentration of nifedipine necessary for significant inhibition of basal activity was 10^{-6} M ($P < 0.001$) with an IC_{50} of 3.4×10^{-5} M. Nifedipine inhibited the increase in enzyme activity over basal attributable to both T_4 and milrinone ($P < 0.001$). The minimum concentrations of nifedipine required for significant inhibition of the T_4 and milrinone effects on enzyme activity were 10^{-5} M ($P < 0.05$) and 10^{-6} M ($P < 0.02$), respectively, with IC_{50} levels of 2.9×10^{-5} M and 9.0×10^{-6} M.

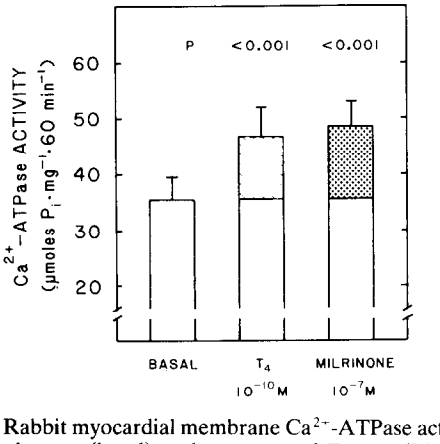

Fig. 1. Rabbit myocardial membrane Ca^{2+}-ATPase activity in the absence (basal) and presence of T_4 or milrinone. Increases in enzyme activity with T_4 (10^{-10} M, cross-hatched area) or milrinone (10^{-7} M, strippled area) were significant ($P < 0.001$) by Student's paired *t*-test. Results shown are means ± SE from three experiments.

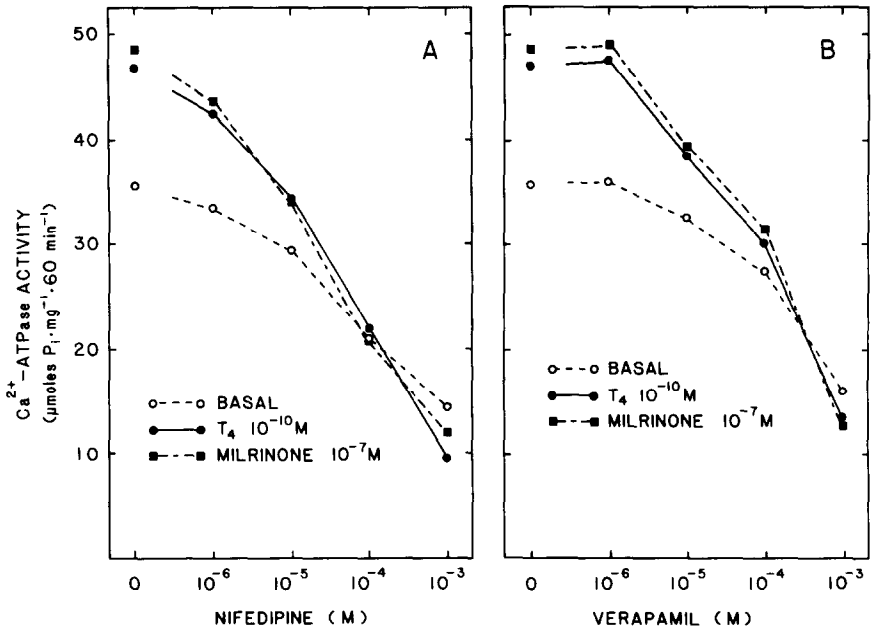

Fig. 2. Effects of nifedipine (A) and verapamil (B), 10^{-6} to 10^{-3} M, on basal, T_4- and milrinone-stimulated Ca^{2+}-ATPase activity in myocardial membranes. Enzyme stimulation attributable to T_4 or milrinone is represented by the increase in enzyme activity over basal, depicted as the area between two curves. Results are means of three experiments.

Verapamil similarly inhibited basal Ca^{2+}-ATPase activity ($P < 0.001$) with a significant effect at 10^{-5} M ($P < 0.05$), and an IC_{50} of 1.5×10^{-4} M (Fig. 2B). The stimulatory effects of T_4 and milrinone on enzyme activity were inhibited by a minimum of 10^{-5} M verapamil ($P < 0.05$) with IC_{50} values of 3.0×10^{-5} M and 5.2×10^{-5} M respectively.

Reversal of nifedipine and verapamil effects by exogenous calmodulin. The inhibition by verapamil (10^{-5} M) of T_4-stimulated Ca^{2+}-ATPase activity was attenuated by the addition of exogenous purified rat testis calmodulin (Fig. 3). Similar results (not shown) were obtained with nifedipine (10^{-5} M). At 40 ng calmodulin/μg membrane protein (1.2×10^{-8} M calmodulin), basal and T_4-stimulated Ca^{2+}-ATPase activity approximated their baselines established in the absence of channel blocker, while the incremental stimulation of enzyme activity by T_4 (cross-hatched area) was fully restored.

Effects of channel blockers on calmodulin binding to myocardial membranes. Total binding of radiocalmodulin to myocardial membranes was $3.96 \pm 0.24\%$. Unlabeled calmodulin reduced [^{125}I]calmodulin binding in a concentration-dependent manner; nonspecific binding of calmodulin accounted for $50 \pm 7\%$ of the total radiolabeled calmodulin bound. Nifedipine and verapamil, in concentrations of 10^{-6} and 10^{-4} M, significantly inhibited the binding of calmodulin to rabbit myocardial membranes ($P < 0.01$, Table 1). The calmodulin inhibitor W-7 (10^{-6} M to 10^{-4} M) inhibited specific calmodulin binding by up to 85%, whereas nifedipine and verapamil (10^{-6} M) inhibited the specific binding of calmodulin by 35 and 55% respectively.

DISCUSSION

The calcium channel antagonists are a structurally and pharmacologically heterogeneous group of compounds which have in common the ability to inhibit sarcolemmal calcium entry into muscle cells. There is evidence that their specific mechanisms of action

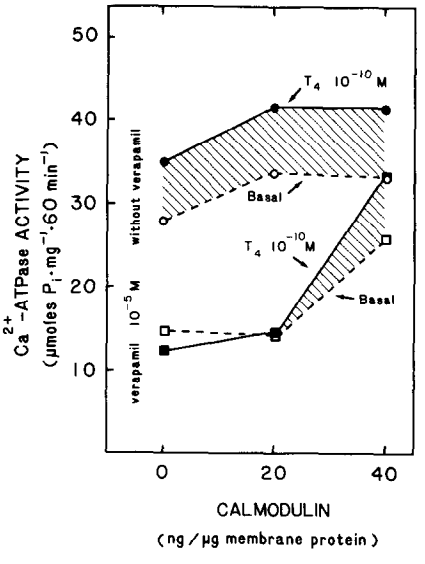

Fig. 3. Effect of exogenous calmodulin on inhibition of basal and T_4-stimulated Ca^{2+}-ATPase activity by verapamil. Results are means of three experiments conducted in duplicate. Enzyme stimulation attributable to T_4 (10^{-10} M) is represented by the increase in enzyme activity over basal (the cross-hatched area between two curves).

Table 1. Effects of nifedipine, verapamil and W-7 on binding of calmodulin to rabbit myocardial membranes

Agent		Total % calmodulin bound to membranes	% Reduction in specific binding*	P†
DMSO (control)	1%	3.96 ± 0.24		
Nifedipine	10^{-6} M	3.28 ± 0.23	35	<0.001
	10^{-4} M	2.88 ± 0.26	55	<0.01
Verapamil	10^{-6} M	2.89 ± 0.16	55	<0.01
	10^{-4} M	2.65 ± 0.15	67	<0.001
W-7	10^{-6} M	2.94 ± 0.26	52	<0.01
	10^{-4} M	2.29 ± 0.10	85	<0.001

Results are means ± SE of three binding experiments, performed in duplicate, using two rabbit heart membrane preparations. Nonspecific binding accounted for 50 ± 7% of the calmodulin that was bound to membranes.

* Calculation is based on 1.96% calmodulin that was specifically bound.

† P was calculated by Student's paired *t*-test, comparing binding in the presence of each compound with binding in the presence of control diluent, 1% DMSO.

and receptor sites may be different [22–24]. Mechanisms of action proposed for this group of drugs, in addition to inhibition of calcium entry, have included stimulation of smooth muscle membrane Na^+,K^+-ATPase [25], and alteration in Ca^{2+} binding by skeletal and cardiac sarcoplasmic reticulum membranes [23]. Recently, several of these agents have been shown to have *in vitro* activity as calmodulin antagonists [12, 26] via a putative direct interaction with calmodulin, causing inhibition of calmodulin-regulated enzyme activity [27]. Mas-Oliva and Nayler [28] have reported inhibition by verapamil of sarcolemmal Ca^{2+}-ATPase activity, a well characterized calmodulin-stimulable enzyme, whereas others have reported increased sarcoplasmic reticulum Ca^{2+}-ATPase activity in skeletal and cardiac muscle with some, but not all, the dihydropyridines [29, 30].

In the present study, we have developed evidence supporting an anti-calmodulin action of verapamil and nifedipine in a rabbit myocardial membrane Ca^{2+}-ATPase system. We have shown previously in the human erythrocyte [2], as well as in rabbit heart membranes [1], that thyroid hormone stimulation of plasma membrane Ca^{2+}-ATPase is a calmodulin-dependent process. In sarcolemma-enriched myocardial membranes, but not in the erythrocyte, milrinone also stimulates this enzyme and the effects of both milrinone and T_4 are inhibited by low concentrations of W-7. This suggests that these agents require calmodulin to express their action on the myocardial enzyme [3]. The inhibitory effects of verapamil and nifedipine on stimulation of sarcolemmal Ca^{2+}-ATPase activity by T_4 and milrinone are shown in the present study to be similar to the effect of W-7. In addition, restoration by calmodulin of thyroid hormone-stimulated Ca^{2+}-ATPase activity in the presence of these calcium channel antagonists provides strong evidence for a specific anti-calmodulin effect of nifedipine and verapamil in this assay system. Addition of calmodulin has also been shown, in red cell membranes, to reverse inhibition of the T_4 effect on Ca^{2+}-ATPase activity by calmodulin inhibitors such as calmidazolium (R24571), W-7, monospecific anti-calmodulin antibody, and bepridil and cetiedil [2, 31, 32]. Although other investigators have described an inhibitory effect of verapamil, but not nifedipine, on Ca^{2+} binding to sarcolemmal membranes [33], our results are consistent with a calmodulin-mediated effect, rather than an alteration of membrane Ca^{2+} binding.

Selected calcium channel blockers have been shown to bind in a Ca^{2+}-dependent manner to calmodulin [34, 35]. Luchowski *et al.* [36] and Johnson [37] have proposed the existence of a calmodulin-like Ca^{2+} receptor in the Ca^{2+} channel; Johnson [38] has shown that calmodulin antagonists can affect dihydropyridine binding to calmodulin. Thayer and Fairhurst [13] have suggested that, at least in the case of the dihydropyridine nitrendipine, the low-affinity, unsaturable nature of the binding of the drug to calmodulin is not pharmacologically significant. Our binding studies presented here suggest that, by combining with calmodulin, nifedipine and verapamil act as calmodulin antagonists in our Ca^{2+}-ATPase system.

We considered the possibility that inhibition of myocardial membrane Ca^{2+}-ATPase activity was secondary to the primary effect of channel blockers, namely, decrease of calcium influx into membrane vesicles, leading to lower intravesicular Ca^{2+} concentrations, and a decrease in plasma membrane Ca^{2+}-ATPase activity. The reversibility of this inhibition of Ca^{2+}-ATPase by calmodulin in the present studies, however, and the effect of channel blockers on Ca^{2+}-ATPase in the red cell (which lacks a voltage-dependent calcium channel) suggest a primary action of these compounds on calmodulin.

The clinical effects of the channel blockers are expressed, it is widely acknowledged, in terms of decreased intracellular Ca^{2+} concentration. Results of the present studies indicate that the action of channel blockers on myocardial membranes is not exclusively at the channel and may, in fact, be partially offset by effects at the calcium pump through calmodulin antagonism. This Ca^{2+}-ATPase action requires micromolar concentrations of channel blockers, whereas the affinities of binding sites for calcium antagonists at the channel are in the nanomolar range. The discrepancy between binding site affinity and the concentrations of channel blockers

required for bioactivity in various models is 100- to 1000-fold, as pointed out by Lee and Tsien [39] and others [40–42]. Concentration by cardiac and smooth muscle of both verapamil and nifedipine *in vitro* has been demonstrated by others [43, 44], an observation which would provide a basis for the discrepancy between site affinity and levels of agents needed for tissue activity.

The membrane preparation utilized in the present studies is enriched in sarcolemma, relative to homogenate, but contains sarcoplasmic reticulum. If, as suggested by Williams and Jones [45], channel blockers have actions at intracellular sites such as the sarcoplasmic reticulum, then their effect on the Ca^{2+}-ATPase enzyme at that site may be relatively more important than their effect on the sarcolemmal Ca^{2+} pump, and potentially of a negatively inotropic nature.

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