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CALMODULIN-STIMULATED PLASMA MEMBRANE (Ca^{2+} + Mg^{2+})-ATPase: INHIBITION BY CALCIUM CHANNEL ENTRY BLOCKERS*

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Abstract—Calcium channel entry blockers representing different structural classes were studied for their effects on human erythrocyte basal and calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase. Effects on the activity of (Mg^{2+})-ATPase and (Na^+ + K^+)-ATPase were also assessed. Of the four Ca^{2+} entry blockers tested, only verapamil and diltiazem specifically inhibited the calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase activity, the basal enzyme activity being unaltered by these drugs. Other membrane-associated ATPases were not affected. Calmodulin concentration effect curves showed the inhibition by verapamil (10^{-3} M) and diltiazem (10^{-3} M) to be non-competitive. This concentration inhibited the calmodulin-dependent increment (5.1 nM calmodulin) of the ATPase activity by 35 and 36% respectively. Similarly, both drugs inhibited the Ca^{2+}-activation process of calmodulin-stimulated activity in a non-competitive manner, decreasing V_{max} by 23 and 17% respectively. Basal (Ca^{2+} + Mg^{2+})-ATPase activity was not affected by verapamil or diltiazem at any calcium concentration. In contrast, cinnarizine non-specifically inhibited all four membrane ATPases including calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase activity at concentrations above $3 \cdot 10^{-6}$ M. Nifedipine was without effect on any of the four membrane ATPases. From this we conclude that certain calcium channel entry blockers can inhibit calmodulin-regulated plasma membrane Ca^{2+}-pump ATPase. Therefore, this identifies an additional functional low affinity receptor in the plasma membrane for some of the calcium channel entry blockers.

A highly regulated Ca^{2+}-pump ATPase in the plasma membrane of human erythrocytes and probably all animal cells is essential to the maintenance of low intracellular Ca^{2+} concentrations [1]. This study describes the effects of certain calcium channel entry blockers, a relatively new and rapidly expanding class of chemically and pharmacologically heterogeneous compounds, on the regulation of this essential Ca^{2+} extrusion mechanism. A primary mode of action of these agents is thought to involve direct inhibition of slow Ca^{2+} influx channels in the plasma membrane and/or to affect Ca^{2+} pools located on these membranes [2]. Recently, however, several of the calcium channel entry blockers were shown to also bind to calmodulin [3, 4] and to interfere with intracellular processes such as calmodulin-dependent stimulation of 3′,5′-cyclic nucleotide phosphodiesterase [5, 6]. Furthermore, Glossmann and coworkers found evidence for low affinity sites associated with the nucleoside carrier in several tissues, including the human red cell [7, 8].

As a result of the apparent involvement of several diverse sites of action of calcium channel entry blockers, we felt that it was important to ask and pursue in a systematic manner questions regarding the possible effects of these drugs on calcium-translocating mechanisms other than the slow calcium channels. The system used here should provide us with at least some answers regarding the effects of calcium channel entry blockers on Ca^{2+}-extrusion and on an additional calmodulin-dependent process. Knowing all primary and secondary sites of action may provide us with a better understanding and means of exploitation of these important therapeutic agents.

MATERIALS AND METHODS

Outdated packed human erythrocytes (7–25 days past the expiration date) were obtained from the local American Red Cross blood banking facility. Verapamil, diltiazem, cinnarizine and nifedipine were supplied by Knoll Pharmaceuticals, Whippany, NJ; Marion Laboratories, Inc., Kansas City, MO; Janssen Pharmaceutica, Inc., New Brunswick, NJ; and Pfizer Inc., New York, NY respectively. Human erythrocyte calmodulin was isolated and purified as noted elsewhere [9]. Crystalline disodium adenosine-5′-triphosphate salt was obtained from Boehringer Mannheim GmbH, West Germany. All other reagents were obtained through the Sigma Co., St. Louis, MO.

Membrane preparations. Packed cells were washed three times in 3 vol. of 154 mM NaCl, and the buffy coat was removed. The cells were hemolyzed in 15 vol. of 20 mM imidazole, 0.1 mM ethyleneglycolbis(amino-ethylether)tetra-acetate (EGTA) buffer, pH 7.4, and then washed an additional two times in 20 mM imidazole, pH 7.4. This membrane pellet was washed with 30 vol. of a buffer (pH 7.1)

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containing 40 mM histidine and imidazole each. This buffer was also used to store the membrane preparation at a concentration of 8–10 mg/ml for up to 5 days without any noticeable loss of activity. Membrane protein was determined by a modified Lowry method described elsewhere [10].

ATPase assays. Incubation media contained 200 μg of membrane protein, 1 mM ATP, 15 mM KCl, 80 mM NaCl, 0.1 mM EGTA, 3 mM $MgCl_2$, 0.1 mM ouabain and various concentrations of free Ca^{2+} and calmodulin as indicated. The final incubation volume was 1 ml. The reactions were initiated by addition of ATP, and incubations were carried out by shaking at 37° for 60 min. Reactions were terminated by the addition of 1 ml of 2% sodium dodecyl sulfate. An automated colorimetric phospho-molybdate complexation method was used to determine inorganic phosphate liberated. Complex formation was measured spectrophotometrically at a wavelength of 750 nm [10].

Operationally defined specific ATPases were determined as follows. Specific (Mg^{2+})-ATPase and $(Na^+ + K^+)$-ATPase activities were measured in the absence of free Ca^{2+} and in the absence of ouabain and free Ca^{2+} respectively. Basal $(Ca^{2+} + Mg^{2+})$-ATPase activities were obtained by addition of $CaCl_2$ to the incubation medium to give free Ca^{2+} concentrations as indicated. Free Ca^{2+} concentrations were determined by a calcium ion-selective Orion electrode in the complete incubation medium at 37°. Calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase activities were determined in the presence of exogenous erythrocyte-derived calmodulin added to the incubation medium. The linearity of the assay under maximal activity conditions was confirmed for a period of 90 min. Where appropriate, drug vehicle controls were performed, and the light-sensitive compound nifedipine was protected from visible light. ATPase activities were expressed as nmoles of inorganic phosphate liberated per mg of membrane protein per min. Specific ATPase activities were calculated from duplicate determinations of two or three independent experiments and reported as the mean ± the standard error of the mean. Error bars are not illustrated when smaller than the symbol size or when interfering with other components of the graph. Significance of differences was determined by comparison of sample means using Student's *t*-test analysis.

RESULTS

Experiments were carried out to systematically study the effects of a representative of each of three structurally diverse groups of channel entry blockers on membrane ATPase activities. In particular, concentration effect relationships of verapamil, diltiazem and cinnarizine on erythrocyte (Mg^{2+})-ATPase, $(Na^+ + K^+)$-ATPase, basal and approximate half-maximally calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase were examined. The large concentration range of 10^{-7} to 10^{-3} M was chosen to ensure detection of possible discrete changes in both therapeutic and pharmacological concentrations. Similarly, a sub-optimal calmodulin concentration of $5.1 \cdot 10^{-9}$ M was chosen to allow for demonstration of potentially inhibitory as well as stimulatory effects of each drug.

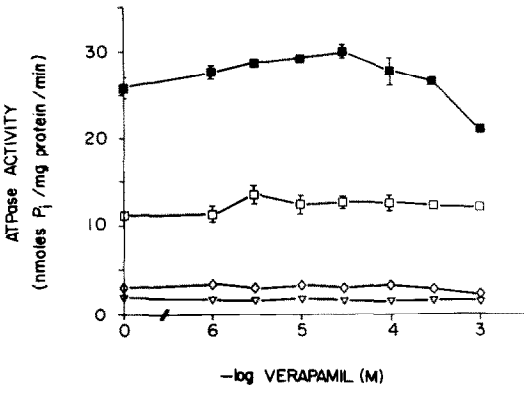

Fig. 1. Effects of verapamil on calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase and other membrane-bound ATPases. Half-maximally calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase ($5.1 \cdot 10^{-9}$ M calmodulin and $1.9 \cdot 10^{-5}$ M Ca^{2+}; ■) and basal $(Ca^{2+} + Mg^{2+})$-ATPase ($1.9 \cdot 10^{-5}$ M Ca^{2+}; □), $(Na^+ + K^+)$-ATPase (◇) and (Mg^{2+})-ATPase (▽) activities were determined in the absence and the presence of various concentrations of verapamil. Specific activities were calculated from two independent experiments with duplicate determinations. Values represent means ± SEM; error bars are omitted where smaller than symbol size.

Figure 1 depicts the effects of verapamil on all four ATPase activities tested. Verapamil appeared to both stimulate and then inhibit the calmodulin-stimulated activity as the concentration of drug was increased from 10^{-6} M to 10^{-3} M. While the stimulation by 3×10^{-5} M verapamil failed to reach stat-

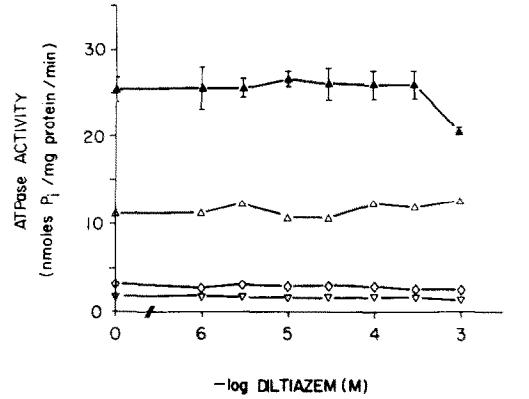

Fig. 2. Effects of diltiazem on calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase and other membrane-bound ATPases. Half-maximally calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase ($5.1 \cdot 10^{-9}$ M calmodulin and $1.9 \cdot 10^{-5}$ M Ca^{2+}; ▲), basal $(Ca^{2+} + Mg^{2+})$-ATPase ($1.9 \cdot 10^{-5}$ M Ca^{2+}; △), $(Na^+ + K^+)$-ATPase (◇) and (Mg^{2+})-ATPase (▼) activities were determined in the absence and the presence of various concentrations of diltiazem. Specific activities were calculated from two independent experiments with duplicate determinations. Values represent means ± SEM; error bars are omitted where smaller than symbol size.

istical significance at the P 0.05 level, inhibition to 79% of control activity by 10^{-3} M verapamil was decreased significantly from control values. Other ATPase activities tested, including basal $(Ca^{2+} + Mg^{2+})$-ATPase activity, were unaffected by verapamil.

Figure 2 demonstrates erythrocyte ATPase effects of diltiazem under identical conditions to those described in Fig. 1. Like verapamil, diltiazem only affected the calmodulin-stimulated ATPase activity and indeed produced an identical degree of inhibition. However, no tendency towards stimulation could be observed with lower concentrations of diltiazem.

Cinnarizine, in contrast to both verapamil and diltiazem, elicited a more potent, although considerably less specific, inhibitory response. As can be seen from Fig. 3, concentrations of cinnarizine above 10^{-6} M not only reduced calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase activity but also interfered with the non-calmodulin-dependent form of this enzyme. Furthermore, the $(Na^+ + K^+)$-ATPase activity and possibly that of the (Mg^{2+})-ATPase were also diminished. At a concentration of 10^{-4} M, cinnarizine completely abolished calmodulin stimulation and inhibited basal $(Ca^{2+} + Mg^{2+})$-ATPase, the $(Na^+ + K^+)$-ATPase and the (Mg^{2+})-ATPase by 52, 40 and 33% respectively (Fig. 3). Inhibition of all calmodulin-dependent and calmodulin-independent ATPase activities by $3 \cdot 10^{-5}$ M cinnarizine and above was found to be decreased significantly from control values in the absence of drug at the P 0.05 level.

Similar experiments to those described above were performed with nifedipine (10^{-7} to 10^{-4} M). Over a large concentration range up to its solubility limits, this compound failed to affect any of the ATPases tested (results not shown).

To further explore the nature of the inhibitory effects of verapamil and diltiazem on calmodulin

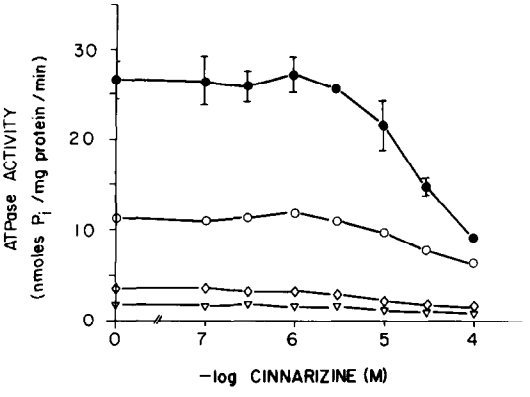

Fig. 3. Non-specific inhibition of all membrane ATPases by cinnarizine. Half-maximally calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase ($5.1 \cdot 10^{-9}$ M calmodulin and $1.9 \cdot 10^{-5}$ M Ca^{2+}; ●), basal $(Ca^{2+} + Mg^{2+})$-ATPase ($1.9 \cdot 10^{-5}$ M Ca^{2+}; ○), $(Na^+ + K^+)$-ATPase (◇) and (Mg^{2+})-ATPase (▽) activities were determined in the absence and the presence of various concentrations of cinnarizine. Specific activities were calculated from two independent experiments with duplicate determinations. Values represent means ± SEM; error bars are omitted where smaller than symbol size.

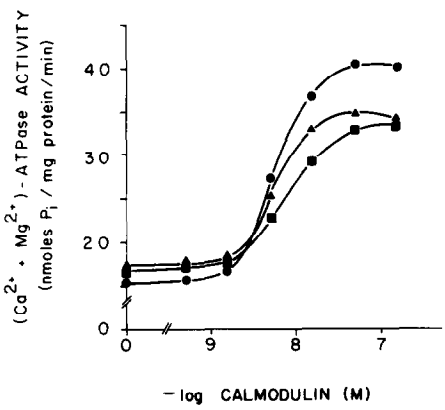

Fig. 4. Inhibitory effects of verapamil and diltiazem on the calmodulin stimulation of $(Ca^{2+} + Mg^{2+})$-ATPase. Calmodulin concentration effect relationship to $(Ca^{2+} + Mg^{2+})$-ATPase activity (●) in the presence of 10^{-3} M verapamil (■) and 10^{-3} M diltiazem (▲). Basal $(Ca^{2+} + Mg^{2+})$-ATPase activities are represented by points on the ordinate. Specific activities reported are the means of three independent experiments with duplicate determinations.

stimulation, we examined the effects of 10^{-3} M verapamil and 10^{-3} M diltiazem on the calmodulin concentration effect relationship with the enzyme. As can be seen in Fig. 4, both drugs antagonized calmodulin stimulation of the enzyme in an apparent non-competitive manner. Verapamil and diltiazem decreased maximal enzyme velocity from 40.5 to 33.5 and 35.0 nmoles $P_i \cdot mg^{-1} \cdot min^{-1}$ respectively. Neither drug significantly changed the apparent K_d or the basal enzyme activity (as shown by symbols on the ordinate). The latter result is consistent with results shown in Figs. 1 and 2.

To confirm the apparent specificity of verapamil and diltiazem towards the calmodulin stimulation, rather than the Ca^{2+} activation of the $(Ca^{2+} + Mg^{2+})$-ATPase, experiments shown in Fig. 5 were carried out. The effects of these drugs on full spectrum Ca^{2+}-activation curves of the calmodulin-activated enzyme were examined. For control purposes, the same experiments were done in the absence of added calmodulin. Both drugs at concentrations of 10^{-3} M reduced the extent to which Ca^{2+} could activate the partially calmodulin-stimulated enzymic activity. At optimal Ca^{2+} concentration of $1.9 \cdot 10^{-5}$ M, maximum activity of 33.8 was decreased by verapamil and diltiazem to 26.0 and 28.2 nmoles $P_i \cdot mg^{-1} \cdot min^{-1}$ respectively. Double-reciprocal conversion of the data revealed no significant change in the apparent $K_{d,Ca}$ for activation.

On the contrary, in the absence of added calmodulin, the Ca^{2+}-activation curves were clearly unaffected by verapamil (10^{-3} M) or diltiazem (10^{-3} M) over the entire range of Ca^{2+} concentrations tested).

In contrast to results shown in Fig. 5, cinnarizine (10^{-4} M) decreased both basal and calmodulin-dependent Ca^{2+} activation (over the same range of Ca^{2+} concentrations as in Fig. 5) of the $Ca^{2+} + Mg^{2+}$)-ATPase and thus reinforces the notion that non-specific membrane perturbation is occurring at all Ca^{2+} and calmodulin concentrations (curves not shown). A summary of the results with

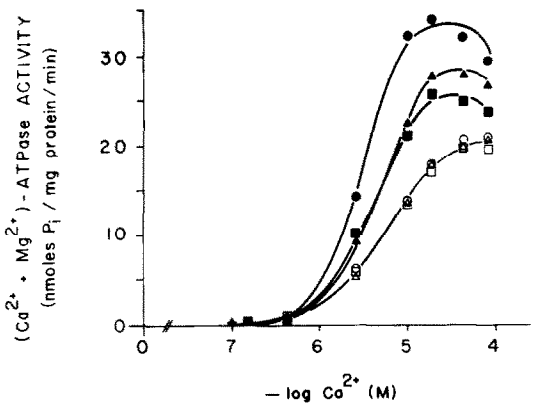

Fig. 5. Effects of verapamil and diltiazem on the Ca^{2+}-activation of basal and calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase. Partially calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase (5.1×10^{-9} M calmodulin, $1.9 \cdot 10^{-5}$ M Ca^{2+}; filled symbols) and basal (Ca^{2+} + Mg^{2+})-ATPase ($1.9 \cdot 10^{-5}$ M Ca^{2+}; open symbols) in the absence (circles) and the presence of verapamil (squares) and diltiazem (triangles), each 10^{-3} M. Activities are reported as the means of three independent experiments with duplicate determinations.

two concentrations of cinnarizine and calmodulin is shown in Table 1.

DISCUSSION

Erythrocyte membrane preparations containing several adenosine triphosphohydrolase activities, including the Ca^{2+}-pump ATPase, are ideal model systems to elucidate functional drug–membrane and drug–membrane–protein interactions. This study focuses on one particular action of an important new class of drugs, generally known as calcium antagonist or, preferably, calcium channel entry blockers. The major portion of a voluminous amount of work investigating the molecular actions of these drugs deals with their primary site of action on the slow, voltage-sensitive calcium channel. To date, several attempts have been made to sort out and classify the various compounds with channel entry blocker activity by means of structural properties, binding characteristics, and pharmacological *in vivo* differences. This subject has been summarized and reviewed in a recent report by Spedding [11]. It has been proposed that, based on structural and functional differences, there are at least three different classes of calcium channel entry blockers. In the present work, we have demonstrated additional direct evidence for the functional heterogeneity among the three proposed classifications. This has been achieved using a unique plasma membrane specific preparation. This model system lacks the classical voltage-sensitive Ca^{2+} channel and in human red cells has no other means of Ca^{2+} extrusion other than the Ca^{2+}-pump ATPase. Thus, as exemplified in this report, the model can be used to answer questions regarding drug effects on (1) the Ca^{2+} pump in particular, (2) calmodulin regulation in general, and (3) other membrane enzymes such as the (Na^+ + K^+)-pump ATPase for identification of possible non-specific ATPase or membrane perturbation effects.

Examples of one type of drug selectivity are demonstrated in Figs. 1, 2, 4 and 5 with verapamil and diltiazem. In each case it can be seen that inhibition of enzymic activity is directed towards the calmodulin-specific portion without affecting the other enzymes measured. On the other hand, cinnarizine (Fig. 3), a compound with structural similarities to phenothiazine-type calmodulin binding drugs, elicited a more potent, although considerably less specific, effect. In the presence of cinnarizine, all ATPase activities were inhibited by concentrations above 10^{-6} M, although that activity by calmodulin appeared to be the most affected.

It is tempting to speculate that the slightly stimulatory effects seen with micromolar concentration of verapamil on the calmodulin-stimulated enzyme (Fig. 1) contribute to the ability of the drug to lower intracellular Ca^{2+}. However, we feel that the stimulation is not well enough defined to warrant the assignment of therapeutic relevance. Clearly, more work is needed along those lines of investigation.

The apparent specificity of verapamil and diltiazem on the calmodulin-dependent activity are supported from data of experiments shown in Figs. 4 and 5. First, in the presence of a fixed amount of free Ca^{2+} ($1 \cdot 10^{-4}$ M), the calmodulin concentration effect relationship was affected non-competitively. Data in Fig. 5 also corroborate the idea of specificity. Here again, verapamil and diltiazem only affected the Ca^{2+} activation of the calmodulin-dependent but not the basal enzyme activity. The non-competitive nature of these drug effects is in contrast to observation made with other calmodulin response modifiers. Phenothiazines [9, 12, 13], the miconazole derivative R 24-571 [14], calmodulin binding proteins [15], compound 48/80 [16] and the naphthalene-

Table 1. Non-selective inhibition of basal and calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase by cinnarizine

	Enzyme activity (nmol P_i liberated $\cdot mg^{-1} \cdot min^{-1}$)		
	Basal (no calmodulin added)	Calmodulin-stimulated (0.087 μg/ml)	(0.867 μg/ml)
Control	20.57 ± 1.77 (0)*	28.51 ± 1.2 (0)	48.87 ± 2.63 (0)
$3 \cdot 10^{-5}$ M Cinnarizine	12.18 ± 1.04 (41)	12.88 ± 0.94 (55)	31.75 ± 3.13 (35)
$1 \cdot 10^{-4}$ M Cinnarizine	5.91 ± 0.00 (71)	7.49 ± 0.66 (74)	18.15 ± 0.06 (63)

Values are means ± SE of two to four independent experiments with duplicate determinations.
* Values in parentheses indicate percent inhibition of control values.

sulfonamide derivatives [17], at least in lower concentrations, tend to inhibit calmodulin-dependent processes in a competitive manner. Furthermore, our data also indicate that the inhibition, albeit modest and at relatively high drug concentrations, is not Ca^{2+} dependent. A proportional amount of inhibition was seen at all Ca^{2+} concentrations tested, and no change in the $K_{d,Ca}$ due to the presence of drug was observed. While there are some reports in the literature describing the binding of channel entry blockers to calmodulin directly [3, 4, 18], our data, notwithstanding their preliminary nature, offer evidence for a different and/or an additional mechanism of action. On the basis of the present results, we tentatively postulate that these drugs do not compete with calmodulin for its binding site on the ATPase and probably do not inhibit the $(Ca^{2+} + Mg^{2+})$-ATPase activation by binding to calmodulin. This would be quite different from the mechanism and site of action of phenothiazine and other hydrophobic compounds in regard to their effects on calmodulin [9, 19–21]. Furthermore, our results indicate that verapamil and diltiazem appear to not interfere with ATP, Ca^{2+} or Mg^{2+} on the basal enzyme, or with Ca^{2+} activation of calmodulin itself. This is based on the lack of interference with the activation of the basal enzyme and no change in the apparent affinity of calmodulin for this enzyme. Rather, it appears that verapamil and diltiazem interact with a site on the basal enzyme and thus interfere with either the calmodulin binding to the ATPase or steps subsequent to the binding process necessary for the activation of the basal activity. This could explain how verapamil and diltiazem inhibit the calmodulin-activated enzyme activity preferentially without affecting basal activity as demonstrated, for instance, by the cluster of symbols on the ordinate (Fig. 4) and the curve with open symbols in Fig. 5.

Our results are in good agreement with those of Scharff and Foder [22] who suggest that there are distinct differences in the mechanisms of calmodulin inhibition by trifluoperazine and verapamil. While these authors found a very small change in the $K_{d,\text{calmodulin}}$ (from 6.0 to 9.3 nM) due to the presence of about 1 mM verapamil, our results showed significant changes only in the maximally achievable activity of the enzyme in the presence of verapamil or diltiazem. No change in the sensitivity of the enzyme to calmodulin in the presence of these compounds suggests a non-competitive inhibition. One must then assume that binding of verapamil, for instance, to calmodulin as reported by others (i.e. Refs. 3 and 4) does not alter the ability of the regulator to activate the $(Ca^{2+} + Mg^{2+})$-ATPase.

The cinnarizine data from Fig. 3 and Table 1, on the other hand, suggest quite a different mechanism. For this drug, which has a much higher lipophilicity (R_f value of 0.14 for the free base) than verapamil and diltiazem (R_f values are 0.33 and 0.32, respectively, [11]), a non-selective site of action in the lipid-bilayer appears to be more plausible. Intercalation in, and derangement of, the lipid-bilayer could explain the observed reduction of all ATPase activities.

In summary, data presented here concur with recent proposals [7, 11] that there exist clear-cut functional differences among the major classes of channel entry blockers. Furthermore, evidence is presented that supports the idea of multiple sites of action of certain calcium channel entry blockers at the level of the plasma membrane. Most likely, drug effects on the calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase described here, because of the large concentrations needed to elicit the inhibitory effects, do not contribute to the therapeutic efficacy of these compounds. Nevertheless, we suggest that in all studies, especially those which employ high doses of benzothiazepine and phenylalkylamine compounds and those which are calmodulin dependent, consideration of the possibility of drug interactions as described here be given.

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