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INHIBITION OF ERYTHROCYTE Ca^{2+}-PUMP BY Ca^{2+} ANTAGONISTS

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Abstract—Inside-out vesicularized membrane fragments from human erythrocytes were prepared to study the effects of various Ca^{2+} channel entry blockers of plasma membrane Ca^{2+} transport and (Ca^{2+} + Mg^{2+})-ATPase activity concomitantly. Verapamil and diltiazem (0.01 to 5 mM) inhibited both (Ca^{2+} + Mg^{2+})-ATPase activity and initial rates of $^{45}Ca^{2+}$ net uptake analogously. In general, the parameter affected most by these drugs, using either Ca^{2+} transport or (Ca^{2+} + Mg^{2+})-5′-adenosine-triphospho-hydrolase (EC 3.6.1.3) ([Ca^{2+} + Mg^{2+}]-ATPase) measurements, was the stimulation by calmodulin. However, the specificity and selectivity of inhibition appeared to be highly concentration and membrane preparation dependent. Verapamil and diltiazem inhibited the calmodulin–Ca^{2+} transport concentration–effect relationship by changing its apparent affinity as well as the maximal velocity of the process. In a "white ghost" membrane preparation, bepridil inhibited calmodulin activation with a high degree of selectivity as opposed to its effects on calmodulin activation in the vesicular preparation. Nifedipine failed to exhibit any specificity and modestly inhibited basal and calmodulin-activated inside-out vesicular Ca^{2+} transport and (Ca^{2+} + Mg^{2+})-ATPase alike. Our results suggest that verapamil, diltiazem and bepridil (0.01 to 0.3 mM), but not nifedipine (1 nM to 0.01 mM), in relatively high concentrations can antagonize the calmodulin-stimulated Ca^{2+}-pump, i.e. the ATPase as well as the transport process. The inhibitors differed with regard to potency, selectivity, and the type of inhibition they produced.

It is well recognized that, in erythrocytes, the (Ca^{2+} + Mg^{2+})-ATPase activity is the enzymatic driving force of the calmodulin-regulated plasma membrane Ca^{2+} extrusion pump. This was first demonstrated by biochemical and biophysical studies [1] and confirmed by a number of means including pharmacological ones where drugs were used to examine both transport and ATPase phenomena [2]. Recent evidence from this laboratory indicates that certain types of calcium channel entry blockers inhibit calmodulin stimulation of (Ca^{2+} + Mg^{2+})-ATPase. Unlike many other drugs, which by virtue of their amphipathic or hydrophobic nature bind to calmodulin and thus prevent it from binding and acting on its target, the calcium entry blockers produce apparent non-competitive inhibition of (Ca^{2+} + Mg^{2+})-ATPase [3, 4]. We postulated that verapamil and diltiazem inhibited calmodulin activation of the (Ca^{2+} + Mg^{2+})-ATPase by acting at a low-affinity site on the enzyme rather than on calmodulin directly, although the latter possibility could not be excluded definitively.

The work described here was prompted to first substantiate the inhibitory effects of verapamil and diltiazem and then to further elucidate the site(s) of action in a preparation with which one can assess both the enzymatic ATPase activity as well as direct effects on the Ca^{2+}-translocating mechanism. Potentially, this approach can provide additional information regarding the sites and mechanisms by which these drugs can modulate Ca^{2+} fluxes across plasma membranes. To this end we describe experiments using inside-out vesicularized (IOV) erythrocyte membrane fragments measuring simultaneously both the biochemical and the biophysical consequences of calcium entry blocker effects on the Ca^{2+} translocating mechanism. This should not only contribute to our understanding of the entire pharmacological spectrum of these drugs, but also identify a novel, potentially useful tool to reversibly inhibit the calmodulin-dependent process by a mechanism(s) other than by occupying the hydrophobic domain on calmodulin which interferes with the regulation of the Ca^{2+}-pump indirectly.

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MATERIALS AND METHODS

Outdated packed human erythrocytes (0–35 days past the expiration date) were supplied by the Western Kentucky Regional Blood Bank facility in Owensboro, KY, or the Red Cross Blood Services Center in Evansville, IN. Verapamil and diltiazem were supplied by Knoll Pharmaceuticals, Whippany, NJ, and Marion Laboratories, Inc., Kansas City, MO, respectively. Nifedipine and bepridil were gifts of the Pfizer and McNeil Pharmaceutical Companies. Crystalline disodium adenosine-5′-triphosphate salt was obtained from Boehringer Mannheim GmbH, F.R.G. Human erythrocyte derived calmodulin and all other reagents were obtained through the Sigma Chemical Co., St. Louis, MO.

Inside-out vesicle preparations. Packed cells were washed three times in 2 vol. of 154 mM NaCl, 0.1 mM ethylene-glycol bis-(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), pH 7.4, at 4°, and the buffy coat was removed by aspiration. The

cells were hemolyzed in 40 vol. of 2.0 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 7.5 to 8.1, containing 0.1 mM EGTA, and centrifuged for 15 min at about 31,000 *g* (r_{max}); then the clear supernatant and a red pellet were removed. Next the remaining fluffy fraction was diluted 1:1 with the hemolyzing buffer and incubated by gentle shaking for 40 min at 37°. The incubation was stopped by placing the membranes on ice and forcefully pushing them four times through a 1-inch 25-gauge hypodermic needle. The processed membranes were then washed with 40 vol. of a solution containing 18 mM KCl, 16.5 mM HEPES, 0.1 mM tris-(hydroxymethyl)aminomethane (Tris), 0.1 mM EGTA, pH 7.5, at 4° and centrifuged for 15 min at 3000 *g* (r_{max}). The pooled vesicles were diluted approximately 1:1 in the same solution and stored on ice at a membrane protein concentration of 8–10 mg/mL. Membrane protein determinations and membrane orientation assessment by acetylcholinesterase accessibility were done essentially according to methods described by Lowry *et al.* [5] and Steck and Kant [6] respectively.

White ghost membrane preparation. A standard, low ionic strength, hemoglobin depleting membrane preparation, as is typically used for ATPase activity measurements, was carried out according to previously described procedures [7].

ATPase assays. Incubation medium contained 200 μg of membrane protein, 1 mM adenosine-5'-triphosphate (ATP), 15 mM KCl, 80 mM NaCl, 0.1 mM EGTA, 3 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.1 mM ouabain, 18 mM histamine and 18 mM imidazole, pH 7.1, at 37°. 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 or 90 min. Reactions were terminated by the addition of 1 mL of 2% sodium dodecyl sulfate. An automated calorimetric phosphomolybdate complexation method was used to determine inorganic phosphate liberated. Complex formation was measured spectrophotometrically at a wavelength of 750 nm [8].

Specific (Mg^{2+})-ATPase and ($Na^+ + K^+$)-ATPase activities were determined in the absence of Ca^{2+} and in the absence of ouabain and Ca^{2+} respectively [8]. Basal ($Ca^{2+} + Mg^{2+}$)-ATPase activities were obtained by addition of $CaCl_2$ to the incubation medium to give a free Ca^{2+} concentration of 2×10^{-5} M. Free Ca^{2+} concentrations were determined by a calcium ion-selective electrode (Orion) in the complete incubation medium at 37° [7]. Calmodulin-stimulated ($Ca^{2+} + Mg^{2+}$)-ATPase activities were determined in the presence of exogenous erythrocyte-derived calmodulin added to the incubation medium. Where appropriate, drug vehicle controls were performed and, if indicated, light-sensitive compounds were protected from visible light. ATPase activities are expressed as nanomoles of inorganic phosphate liberated per milligram of membrane protein per minute. Specific ATPase activities were calculated from duplicate determinations of two or three independent experiments and reported as the mean ± SE. The significance of differences was determined by comparison of sample means using Student's *t*-test analysis.

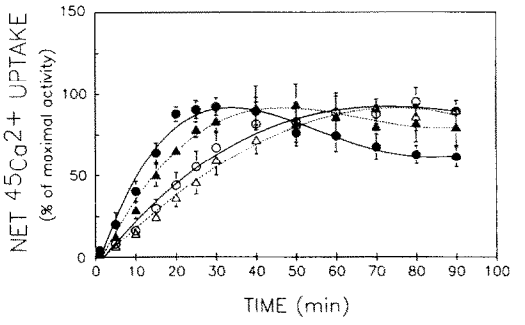

Fig. 1. Effects of verapamil on long-term accumulation of $^{45}Ca^{2+}$ into IOV membrane fragments. Presented is a time-course of basal and calmodulin (0.1 μg/mL) stimulated Ca^{2+} uptake (open and closed circles respectively) in the absence and the presence of 0.3 mM verapamil (open and closed triangles). The incubation conditions were as described under Materials and Methods with the exception of 2 μM $CaCl_2$ and 3 mM ATP additions in a 1 mL total incubation volume, stirred only immediately before sampling at 1- and subsequent 5-min intervals. Values are the means ± SD from five different IOV preparations ranging from 30.2 to 44% inside-out orientation. One hundred percent represents the normalized point of the highest accumulation in the calmodulin control curve between 20 and 40 min and has an absolute mean value of 4.13 ± 0.30 nmol $^{45}Ca^{2+}$/mg IOV protein, N = 5.

$^{45}Ca^{2+}$ net uptake into IOV membrane fragments. Basic transport incubation medium (0.5 or 1.0 mL) contained 18 mM imidazole, 18 mM histidine, 15 mM NaCl, 100 mM KCl, 3 mM $MgSO_4$, 0.1 mM ouabain, 20 μM $CaCl_2$ (sp. act. 0.047 to 0.051 mCi/mg) and 100 μg/mL of membrane protein, pH 7.1. Drugs and calmodulin were added and preincubated for 10–15 min before the addition of substrate. Transport was initiated with the timed addition of prewarmed ATP (1 mM, 37°). At timed intervals of 1, 2 and 3 min (or as indicated), 50-μL aliquots were removed from the stirred incubation vessel and diluted into 60 vol. of an ice-cold stopping solution containing 40 mM Tris, 40 mM glycylglycine, 0.1 mM $MgCl_2$ and 3 mM $CaCl_2$, pH 7.1. The quenched vesicles were trapped on a 0.45 μm, 25 mm membrane filter (Gelman, Metricel GA-6) under 15 psi negative pressure. The filters were immersed in a complete counting mixture (RPI 3a70B) and measured in a Beckman LS 7500 scintillation spectrophotometer.

RESULTS

Long-term basal and calmodulin-stimulated Ca^{2+} accumulation into IOV membrane fragments from outdated red cells in the absence and presence of verapamil. Figure 1 shows $^{45}Ca^{2+}$ accumulation into IOV membrane fragments over a prolonged (90 min) time period. In the presence of 2 μM $^{45}Ca^{2+}$ and in the absence of any added calmodulin (basal), counts accumulated in an apparent linear fashion for about 30 min, and gradually leveled off to a steady-state level between 60 and 90 min. With the addition of 0.1 μg/mL calmodulin, counts accumulated at 2.6

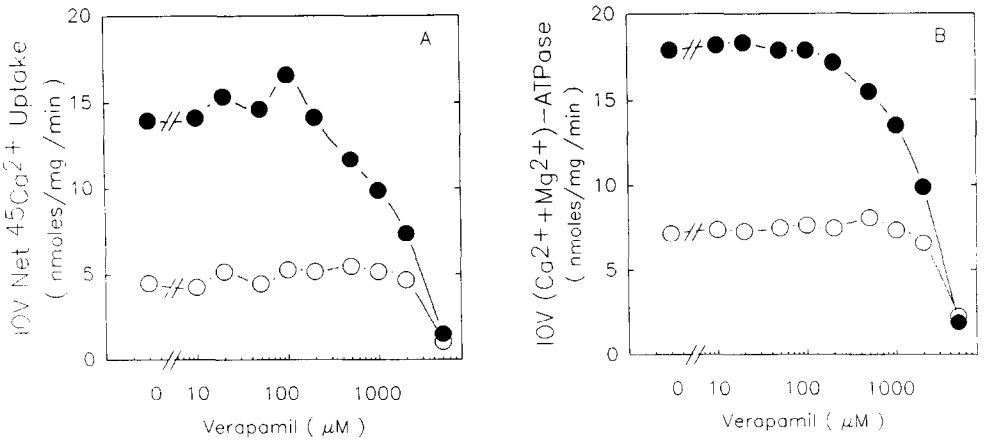

Fig. 2. Concurrent inhibition of IOV (Ca^{2+} + Mg^{2+})-ATPase and net $^{45}Ca^{2+}$ uptake by verapamil. Initial Ca^{2+}-transport rates (0–3 min) (A) and (Ca^{2+} + Mg^{2+})-ATPase activity measurements (90-min incubation) (B) in the absence and the presence of verapamil ranging in concentrations from 0.01 to 5.0 mM are shown. Open and closed symbols are rates of activities in the absence (○) and the presence (●) of 0.1 μg/mL calmodulin respectively.

times the basal rate, reaching a maximum level of 3.1 nmol $^{45}Ca^{2+} \cdot mg^{-1}$ IOV protein at about 20 min from which it gradually declined to roughly 70% of the steady state at 90 min. Initial, nominally linear rates of uptake were 60 pmol $^{45}Ca^{2+} \cdot$(mg IOV protein)$^{-1} \cdot min^{-1}$ for basal uptake and 155 pmol $^{45}Ca^{2+} \cdot$(mg IOV protein)$^{-1} \cdot min^{-1}$ for the calmodulin-stimulated rate. The rate of passive leakage into the vesicles in the absence of ATP over a 60-min period in one experiment with a 10 times greater outside concentration of $^{45}Ca^{2+}$ of 20 μM was 0.17% of ATP-dependent basal uptake (not shown). Also shown in Fig. 1 are the effects of 3×10^{-4} M verapamil on long-term uptake rates of basal and calmodulin-stimulated Ca^{2+} net uptake. Calmodulin-stimulated rates of uptake were decreased on the average of 112 pmol $^{45}Ca^{2+} \cdot$(mg IOV protein)$^{-1} \cdot min^{-1}$ or 72% of control. Basal uptake activity over a 20-min range at this concentration of verapamil dropped to 89.6% of control.

Analogous inhibition of (Ca^{2+} + Mg^{2+})-*ATPase and* Ca^{2+} *transport in IOV membrane fragments by verapamil and diltiazem.* The following transport data presented in this report are derived from the same type of experiments as above with the exception that these short-term experiments were run over a 3-min period in the presence of standard amounts of 1 mM ATP, 0.1 μg/mL of calmodulin (a roughly half-maximally activating concentration) and 20 μM $^{45}CaCl_2$. All initial transport rates, calculated from a linear least squares fit, are based on counts accumulated and corrected for the percentage of inside-out oriented vesicles as assessed by acetylcholinesterase accessibility. Figure 2 demonstrates the concurrent inhibition of IOV (Ca^{2+} + Mg^{2+})-ATPase and net $^{45}Ca^{2+}$ uptake by verapamil. Both Ca^{2+}-transport and (Ca^{2+} + Mg^{2+})-ATPase activity measurements in the absence and the presence of calmodulin and 20 μM Ca^{2+} were affected by verapamil. However, it appeared as if the calmodulin-stimulated portion of the transport and the ATPase activity were antagonized preferentially in a concentration range of 0.2 to 2.0 mM verapamil. The high concentration of 5 mM verapamil basically abolished any type of Ca^{2+} uptake activity. Note the high degree of congruency between the shapes and rates of Ca^{2+}-transport and (Ca^{2+} + Mg^{2+})-ATPase activity. From experiments similar to those depicted in Fig. 2, a summary of inhibition of calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+}-transport activities by verapamil and diltiazem is presented in Fig. 3. Again, a good agreement exists between the degree of inhibition conferred by verapamil toward ATPase and transport activities (panels A and B). Furthermore, the figure also shows that diltiazem affected calmodulin stimulation of transport and ATPase activities in a similar fashion. Half-maximal inhibition of calmodulin stimulation of both processes and by both drugs was in the range of 0.8 to 1.3 mM (panels C and D).

Non-surmountable antagonism of Ca^{2+} *transport into IOV membrane fragments by verapamil and diltiazem.* A concentration–effect relationship analysis of calmodulin activation of Ca^{2+} transport, shown in Fig. 4, indicates an affinity constant of 4.4×10^{-9} M which is in good agreement with affinity constants of other calmodulin-activated processes. The Ca^{2+}-transport stimulation by calmodulin was antagonized in an apparent non-competitive manner showing decreases in maximal velocity of about 40% by 10^{-3} M verapamil and 10^{-3} M diltiazem respectively. Based on a molecular weight of 16,723 daltons, the apparent dissociation constant for calmodulin was increased from 3.6 to 4.2 nmol/L for diltiazem and for verapamil. A 3 mM concentration of verapamil completely prevented stimulation by calmodulin in concentrations as high as 0.866 μg/mL.

Comparison of structurally different Ca^{2+} *channel entry blockers in white ghost membrane and in IOV membrane preparation.* Table 1 shows the effects of

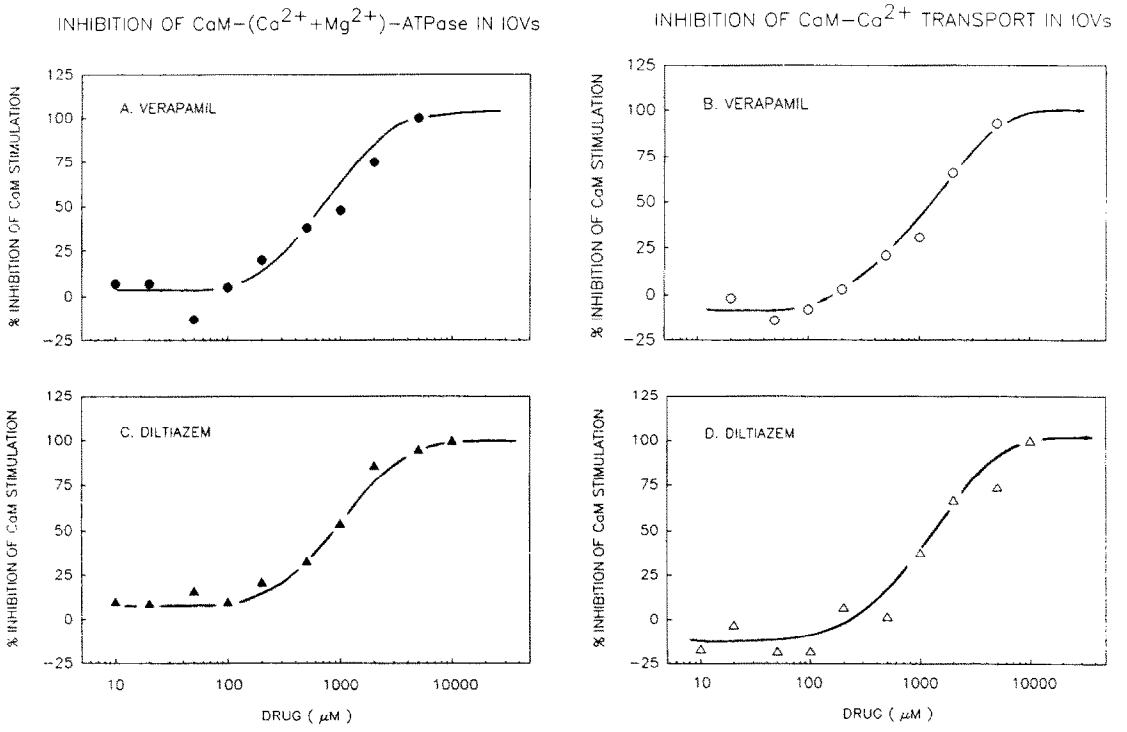

Fig. 3. Summary of calmodulin (CaM) antagonism by verapamil and diltiazem. (A and B): Inhibition of calmodulin (0.1 μg/mL) stimulated portion of (Ca^{2+} + Mg^{2+})-ATPase (●) and Ca^{2+} transport (○) into inside-out vesicularized membrane fragments (IOVs) by verapamil respectively. (C and D): Equivalent experiments with diltiazem as the inhibitor. The normalized 100% value (ordinate) corresponds to complete inhibition of the calmodulin (0.1 μg/mL) stimulated $^{45}Ca^{2+}$ net uptake rate. Data are the means of three to five independent experiments from three different IOV preparations.

four structurally different Ca^{2+} channel entry blockers on Ca^{2+}-pump and ATPase activities in two different membrane preparations. Summarized are

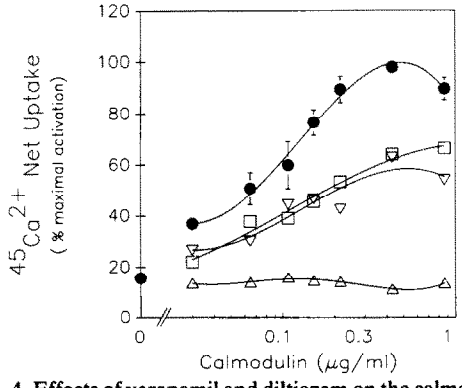

Fig. 4. Effects of verapamil and diltiazem on the calmodulin activation of the Ca^{2+}-pump. Concentration–effect relationships of calmodulin stimulation of Ca^{2+} net uptake rate in the absence (●) and the presence of 10^{-3} M (▽) and 3×10^{-3} M verapamil (△) and 10^{-3} M diltiazem (□) are shown. Data are normalized to the calmodulin control curve (●) with its highest value of accumulation set at 100% representing 11.41 nmol $^{45}Ca^{2+}$/mg/min taken up. Values in the control curves are means ± SE, N = 5.

normalized values of the effects of each compound on both the basal (Ca^{2+} + Mg^{2+})-ATPase activity and on basal Ca^{2+} transport activity as well as the calmodulin-stimulated portion of these activities. Verapamil and diltiazem showed uniform effects in either preparation with corresponding specificity ratios ranging from 1.33 to 1.39. Nifedipine at 10 μM, the highest concentration possible because of solubility limits, afforded no inhibition of either basal or calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase activity in regular white ghost membranes. However, this drug clearly inhibited IOV Ca^{2+}-pump and ATPase activities but, in contrast to the former compounds, without any selectivity. In contrast, 100 μM bepridil, almost completely antagonized the calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase activity with a high degree of selectivity (Fig. 5 and Table 1; B/C ratio >24) in the white ghost membrane preparation, but only modestly inhibited the IOV preparation and with considerably less specificity (Fig. 6). Thus, bepridil did not inhibit basal ATPase activity in regular membranes, it clearly affected vesicular basal (Ca^{2+} + Mg^{2+})-ATPase and transport activities. Apparently, verapamil and diltiazem have a high degree of congruency in both types of membrane preparations,

Table 1. Comparison of inhibitory effects of Ca^{2+} channel entry blockers on white ghost (Ca^{2+} + Mg^{2+})-ATPase and IOV (Ca^{2+} + Mg^{2+})-ATPase/Ca^{2+} transport activities

	% Activity of control without drug								
	Ghost (Ca^{2+} + Mg^{2+})-ATPase			IOV (Ca^{2+} + Mg^{2+})-ATPase			$^{45}Ca^{2+}$ uptake		
Drug (μM)	Basal	CaM	(B/C)	Basal	CaM	(B/C)	Basal	CaM	(B/C)
Verapamil (1000)	104 ± 8	77 ± 1*	1.35	104 ± 4	78 ± 1	1.33	109 ± 14	80 ± 7	1.36
Diltiazem (1000)	106 ± 1	76 ± 2*	1.39	100 ± 3	72 ± 2	1.39	109 ± 13	80 ± 12	1.36
Nifedipine (10)	107 ± 4	102 ± 2	1.05	76 ± 12	76 ± 14	1.00	81 ± 7	82 ± 6	0.99
Bepridil (100)	97 ± 9	4 ± 5*	24.2	77 ± 6	54 ± 11	1.43	80 ± 9	36 ± 6	2.22

Basal (B) and CaM (C) represent activities in the absence and the presence of 0.1 μg/mL or 0.087 μg/mL(*) calmodulin respectively. Activities are expressed as mean ± SE percentages of values in the absence of drug from three to five independent experiments. B/C is the selectivity ratio of inhibition. Data are normalized where 100% represents the activity of each condition in the absence of drug. Absolute values of the three preparations are within the range of activities reported in Figs. 4–6.

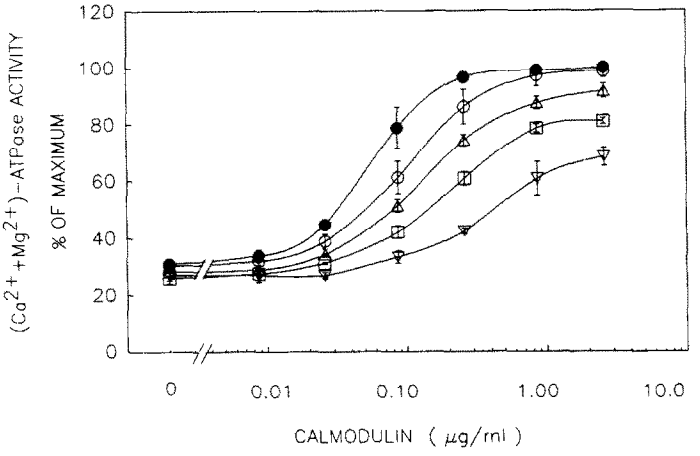

Fig. 5. Bepridil inhibition of calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase in ghost membrane preparations: Calmodulin concentration–effect relationship expressed in percent of maximal enzyme stimulation. Bepridil: (●) control; (○) 10 μM; (△) 30 μM; (□) 50 μM; and (▽) 100 μM. One hundred percent activity is defined as P_i liberated (54.9 nmol P_i/mg protein/min) in the presence of 2.6 μg/mL calmodulin. Results shown are the means ± SE of three independent experiments.

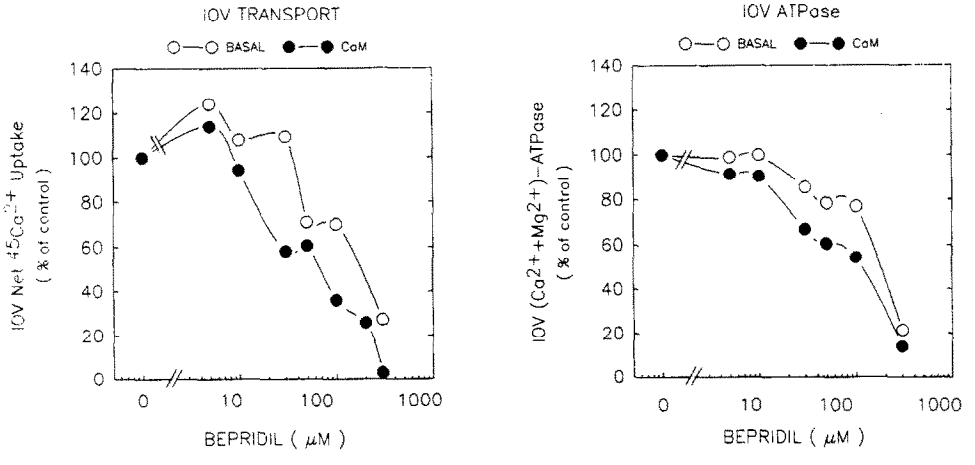

Fig. 6. Relative lack of selective bepridil inhibition of calmodulin stimulation of IOV Ca^{2+} transport and ATPase activities. Basal and calmodulin (0.1 μg/mL) stimulated IOV transport (left panel) and concomitant ATPase measurements (right panel) are presented. The IC_{50} values for the transport process were 60 μM (CaM) and 130 μM (basal) and for the ATPase measurements 100 μM (CaM) and 170 μM (basal) respectively. Results shown are the means of three to five independent experiments. One hundred percent basal and calmodulin-stimulated transport values are 4.92 and 14.21 nmol $^{45}Ca^{2+}$/mg/min respectively. One hundred percent basal and calmodulin-stimulated IOV ATPase activity values are 8.37 and 20.46 nmol P_i/mg/min respectively.

whereas nifedipine and bepridil affect Ca^{2+} transport and (Ca^{2+} + Mg^{2+})-ATPase activities with different sensitivity and specificity depending on what type of membrane preparation is used.

DISCUSSION

A variety of structurally heterogeneous compounds, commonly referred to as Ca^{2+} antagonists, are thought to mediate negative ino-, chrono- and dromotropic cardiac effects, vasodilation, and protection against structural damage in response to a cellular Ca^{2+} overload [9]. While these compounds appear to oppose entry and consequences of intracellular Ca^{2+}, they can, at the same time, also interfere with a number of non-L type Ca^{2+} channel sites including the very mechanism that actively extrudes Ca^{2+} from the cell interior [10]. To describe fully a transport system and understand its physiological implications, including how drugs may affect it, it is imperative that both the biochemical and the biophysical aspects of the transport mechanism be studied, preferably under closely matched experimental conditions. It is under these circumstances that pharmacological evidence can be applied to bear on questions of symmetry of the processes, contributions of passive fluxes to the overall process, stoichiometry and also how the drugs themselves affect the translocation mechanism.

Using a combined, closely matched experimental approach, we have attempted to delineate the effects of Ca^{2+} antagonists simultaneously on both activities of the plasma membrane Ca^{2+}-pump. Typically, (Ca^{2+} + Mg^{2+})-ATPase activities are determined over a 60- or 90-min period, whereas Ca^{2+} transport measurements usually are a short-term measurement to remain within the linear portion of the transport curve, thus limiting the contributions of back flux. Since our previous work on Ca^{2+} antagonist effects on (Ca^{2+} + Mg^{2+})-ATPase was done with white ghost membranes over prolonged periods of time [3, 4], the experiments shown in Fig. 1 were carried out to examine the effects of verapamil on IOV Ca^{2+} transport over a comparable time span. As expected from ATPase and shorter 0- to 3-min transport experiments, the rate of accumulation to maximum was reduced appreciably by 3×10^{-4} M verapamil and also seemed to blunt the roll-over phenomenon seen in the presence of calmodulin. Whether the roll-over was due to calmodulin antagonism or to a direct effect on the membrane (e.g. a membrane-stabilizing effect) is not clear at this time. Basal activity was not reduced significantly at this same concentration of verapamil but, interestingly enough, achieved a higher level at 90 min, as did the control curve. This may be, in part, because the basal uptake curve lacks the gradual decline after reaching its maximum which is characteristic of the uptake curve in the presence of calmodulin. Alternatively, the roll-over and the somewhat higher plateau may be explained by a Ca^{2+} or Ca^{2+}–calmodulin induced increased passive leak out of the vesicle which apparently is also sensitive to inhibition by verapamil. The possibility of a passive back leak is strengthened by the observation that similar red cell vesicles are estimated to accumulate, over a 3-min period, intracellular Ca^{2+} concentrations as high as 2.5 mM [2].

Blocking passive Ca^{2+} entry in intact erythrocytes and Ca^{2+} exit in IOV membrane fragments by these drugs is a likely possibility since we know from recent hemorheological studies that other Ca^{2+} antagonists, such as bepridil, which are also calmodulin antagonists, can inhibit passive Ca^{2+} leaks across the red cell plasma membrane [11, 12]. Thus, verapamil appears to affect predominantly the rate at which Ca^{2+} is accumulated, rather than the extent to which these vesicles are capable of trapping the cation.

A high degree of congruency of Ca^{2+} transport and (Ca^{2+} + Mg^{2+})-ATPase activities is shown in Figs. 2 and 3. Verapamil and diltiazem affected both parameters in an analogous manner with an IC_{50} for the inhibitors of roughly 10^{-3} M. Up to this antagonist concentration selectivity for inhibition of the calmodulin-stimulated portion was reasonably well preserved but rapidly deteriorated with even higher concentrations. From this it appears as if these compounds do not uncouple but inhibit enzymic activity by at least two different modes of action, one of which involves antagonism of calmodulin activation. As in the case of phenothiazine effects on (Ca^{2+} + Mg^{2+})-ATPase activities [13, 14], the present results with verapamil and diltiazem also suggest calmodulin-specific as well as direct enzyme inhibitory actions. This is contrasted by two other Ca^{2+} antagonists, nifedipine on the one hand, which showed no selectivity in either of the preparations tested, and bepridil which at 100 μM quite readily antagonized the effects of calmodulin specifically, particularly so in the white ghost preparation (Table 1). It is not clear why in the vesicle preparation basal activities of (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+}-transport appeared to be affected much more severely by bepridil, thus diminishing the specificity ratio considerably. The difference in Ca^{2+} entry blocker effects, particularly nifedipine and bepridil effects on the ghost preparation and on IOV ATPase activities, compared to the similarity of effects of verapamil and diltiazem, may well be an indication of the susceptibility of the vesicular preparation to drugs with a high degree of hydrophobicity. Notwithstanding, it also points out the remarkable lack of adverse effects of the diphenylalkylamine and benzothiazepine derivative which may explain in part their relative lack of *in vivo* toxicity.

The complex nature of inhibition of the Ca^{2+}-pump by verapamil and by diltiazem is further illustrated by examining the concentration–effect relationship of the calmodulin activation of Ca^{2+} transport (Fig. 4). These transport data, showing mixed kinetics, are in good agreement with data reported for calmodulin stimulation from our earlier work examining the effects of these drugs on (Ca^{2+} + Mg^{2+})-ATPase activity [3, 4]. As has been pointed out in recent reviews on drug interactions with the Ca^{2+} translocation mechanisms [15, 16], data presented here corroborate that there are several possible modes of action that lead to pharmacological modification of the plasma mem-

brane Ca^{2+}-pump. It appears that certain drugs, such as bepridil, can affect the same pump mechanism in more than one way, depending on the dose and the experimental preparation used. While membrane stroma and vesicle preparation are important tools in identifying drug interactions with the Ca^{2+}-pump mechanism initially, they have limited usefulness in delineating the exact nature of the interaction of drugs with the Ca^{2+}-pump. Rather, these studies will need to be extended by identifying and characterizing the binding sites for these drugs on the purified pump protein outside its membrane environment. This should eventually, together with subsequent reconstitution of the protein, provide a rational basis from which other specific and hopefully more selective inhibitors can be developed.

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