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SLOW CALCIUM CHANNEL BLOCKERS AND CALMODULIN

EFFECT OF FELODIPINE, NIFEDIPINE, PRENYLAMINE AND BEPRIDIL ON CARDIAC SARCOLEMMAL CALCIUM PUMPING ATPASE

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Abstract—The effect of four slow Ca^{2+} channel blockers (felodipine, nifedipine, prenylamine and bepridil) that possess the ability to bind to calmodulin (CaM)§ and to inhibit myosin light chain kinase (MLCK) on CaM-regulated Ca^{2+} pumping ATPase of cardiac sarcolemma (SL) and brain cyclic AMP phosphodiesterase (PDE) was studied. The ability of these drugs to inhibit Ca^{2+} pumping ATPase correlated with their inhibitory effect on CaM-activated Ca^{2+}-dependent PDE. Nifedipine was unable to inhibit markedly both enzymes. Prenylamine also was a weak inhibitor, which was unexpected because of its CaM binding potency. Felodipine (10–50 μM) and bepridil (50 μM) markedly reduced activities of SL Ca^{2+} pumping ATPase and PDE. Striking differences were, however, demonstrated when Ca^{2+} and CaM concentrations, respectively, were increased. Previously it was reported that inhibition of the SL Ca^{2+} pumping ATPase by the CaM antagonist calmidazolium could be overcome by increasing Ca^{2+} concentrations (J. M. J. Lamers and J. T. Stinis, *Cell Calcium* **4**, 281–294, 1983). Felodipine (10–50 μM) in the present study, appeared to be equipotent with calmidazolium in reducing Ca^{2+} pumping ATPase, but increasing Ca^{2+} up to 12.2 μM could not counteract this effect. Felodipine (2–10 μM) also inhibited brain PDE noncompetitively with respect to CaM contrary to the competitive effectors calmidazolium and bepridil. On the other hand, bepridil (10–20 μM) decreased or increased Ca^{2+} pumping ATPase activity depending on the Ca^{2+} concentration (0.29 and 12.2 μM, respectively) used. These findings suggest at least two types of CaM antagonists, which can be discriminated on basis of their inhibition patterns of PDE and heart SL Ca^{2+} pumping ATPase.

Slow Ca^{2+} channel blockers (also termed Ca^{2+} entry blockers or Ca^{2+} antagonists) represent a broad class of organic compounds which inhibit the movement of Ca^{2+} through Ca^{2+} selective channels [1]. Several lines of evidence suggest that these drugs are not only active at the extracellular side of the sarcolemma, but also act intracellularly [2–6]. It seems reasonable to expect that interference with Ca^{2+}-CaM-mediated processes plays a role in their intracellular actions. Indeed, a number of slow Ca^{2+} channel blockers, felodipine, nitrendipine, nifedipine, verapamil, prenylamine and bepridil are capable of binding and inhibiting CaM [3, 5, 7–10]. These selected drugs, like the whole group of Ca^{2+} antagonists, are structurally and pharmacologically different which indicates that differences in the mode of action perhaps not only exist at the slow Ca^{2+} channel, but also at the intracellular site of action.

Ca^{2+} binding to CaM exposes hydrophobic sites that can bind inhibitory ligands such as calmidazolium, trifluoperazine and several slow Ca^{2+} channel blockers [11–13]. Calmidazolium, felodipine, prenylamine and verapamil bind Ca^{2+} dependently to CaM with K_d's of 2.5 nM, 3, 0.5 and 30 μM, respectively [7]. It is generally believed that the hydrophobic drug binding site on CaM, exposed by occupation of the Ca^{2+} sites, also represents the binding site for CaM binding proteins and CaM-activated enzymes [11–13], such as MLCK and cAMP-PDE. It is, however, not a general property of all CaM-activated enzymes as, e.g., the SL Ca^{2+} pumping ATPase and phosphorylase *b* kinase have CaM subunits which remain tightly bound to the enzyme molecules after isolation of microsomal respectively supernatant fractions [14–17]. Hypotonic and subsequent hypertonic treatment in the presence of a Ca^{2+} chelating agent is required to remove CaM from the Ca^{2+} pumping ATPase in cardiac SL vesicles [18]. We reported earlier that inhibition of SL Ca^{2+} pumping ATPase by calmidazolium and trifluoperazine could be overcome by increasing Ca^{2+} concentrations [14, 17]. This finding offered the first indication that the SL Ca^{2+} pumping ATPase is regulated by endogenous CaM

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§ Abbreviations: SL, sarcolemma; SR, sarcoplasmatic reticulum; PDE, phosphodiesterase; CaM calmodulin; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis (*p*-amino ethylether)-*N*,*N*,*N*′,*N*′ tetraacetic acid; MOPS, (*N*-morpholino)-propanesulfonic acid; SDS-PAGE; SDS-polyacrylamide gelelectrophoresis; Ca^{2+} pumping ATPase, Ca^{2+}-stimulated Mg^{2+}-dependent ATP hydrolyzing activity; Tris–HCL, Tris(hydroxymethyl)aminomethane hydrochloride; MLCK, myosin light chain kinase; cAMP, cyclic AMP.

[14, 17–19]. It was most convincingly demonstrated by purification of the CaM-depleted SL enzyme on a CaM-affinity chromatography column [18]. At present there are only two studies on the effect of Ca^{2+} antagonists on the SL Ca^{2+} pumping ATPase [20, 21]. The results are, however, conflicting as inhibition [20] as well as activation [21] of the enzyme has been shown. Other studies dealing with SR Ca^{2+} pumping ATPase have demonstrated that verapamil, diltiazem, nisoldipine and felodipine all were stimulatory in a concentration range 20–40 μM [22, 23]. Slow Ca^{2+} channel blockers of different chemical structures, undergoing complex chemical–membrane interactions may prove to be valuable as probes for studying the molecular mechanism(s) involved in the regulation of Ca^{2+} pumping ATPases.

Recent studies with microsomal membranes from guinea pig intestinal smooth muscle and rabbit ventricle revealed that the high-affinity dihydropyridine binding sites are proteins with a hydrophobic domain, very similar to that exposed by complexation of CaM with Ca^{2+} [24, 25]. It represents, in fact, the first indication for drug binding to a CaM-like protein present in its physiological environment. CaM antagonists inhibit high affinity dihydropyridine binding in the order of potency calmidazolium > trifluoperazine > chlorpromazine, which correlates quite well with the potency of these drugs to inhibit CaM-dependent processes [24, 25]. On the basis of electrophysiological data Johnson [5, 26] postulated that the slow Ca^{2+} channel may contain an intracellularly localized CaM-like subunit protein which functions as the Ca^{2+} receptor for the Ca^{2+}-dependent closing gate [26]. In this hypothesis CaM and Ca^{2+} antagonists increase the Ca^{2+} affinity of the intracellular Ca^{2+}-dependent closing gate of the slow Ca^{2+} channel. Thus, in view of the apparent analogy of CaM-like protein representing the intracellular receptor site for Ca^{2+} of the cardiac SL Ca^{2+} channel and Ca^{2+} pumping ATPase, the protein may also turn out to be a common target of Ca^{2+} antagonists.

To gain further insight into the anti-CaM action of some slow Ca^{2+} channel blockers, their effects were studied on the SL Ca^{2+} pumping ATPase. As the latter enzyme represents an example of a membrane-bound enzyme, the same drugs are compared for their ability to inhibit the soluble brain PDE enzyme.

MATERIALS AND METHODS

Materials. Felodipine was a gift from A. B. Hässle (Mölndal, Sweden), nifedipine from Bayer Pharma (Wuppertal, West Germany), prenylamine-lactate from Hoechst Holland N.V. (Amsterdam, The Netherlands) and bepridil from Organon Company B.V. (Oss, The Netherlands). Radioactive substances, [γ-^{32}P]-ATP and [adenine-U-^{14}C]-cyclic AMP were obtained from Amersham International PLC (Amersham, U.K.) and CaM was obtained from Boehringer (Mannheim, West Germany). Calmidazolium was supplied by Janssen Pharmaceuticals (Beerse, Belgium). All other chemicals were obtained from either Merck (Darmstadt, West Germany) or Boehringer (Mannheim, West Germany). CaM-deficient PDE, purified from dog brain and with an activity of approximately 100 mU/mg protein [cf. ref. 27], was kindly supplied by Dr. H. R. Van Belle, Janssen Pharmaceuticals (Beerse, Belgium).

Isolation of purified sarcolemma (SL). SL was isolated from porcine heart according to the procedure described by Reeves and Sutko [28], the only difference being the addition of protease inhibitor phenylmethylsulfonyl fluoride to all media in 0.5 mM concentration. The preparation was highly enriched in SL as was revealed by the high specific activity of Na^+/K^+-ATPase [29]. Cross contamination with SR membranes was minimal which was verified with Na^+- and Ca^{2+}-dependent ^{32}P incorporation into 120 kDa proteins separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE [see refs. 4, 29, 30]). After isolation, the vesicles were suspended in 160 mM KCl and 20 mM 3-(*N*-morpholino)-propane sulfonic acid (MOPS), pH 7.4 at a protein concentration of about 1 mg/ml, immediately frozen in liquid nitrogen and stored at −80°.

Assay of SL Ca^{2+} pumping ATPase. Ca^{2+}/Mg^{2+}-ATPase activity was determined with the [$^{32}P_i$]-molybdate extraction method and [γ-^{32}P]-labeled ATP as a substrate [4, 29, 30]. Ca^{2+}-CaM antagonists were added to plastic Eppendorf tubes containing 0.2 ml reaction mixture of 5 mM $MgCl_2$, 100 mM KCl, 50 mM Tris–maleate (pH 6.8, 37°), 0.1 mM [γ-^{32}P]-ATP (10 mCi/mmol). Free Ca^{2+} (0.29 or 12.2 μM as indicated) was controlled in the sub-μM range by buffering with 100 μM ethylene glycol bis (*p*-amino ethylether-*N*,*N*,*N*′,*N*′,-tetraacetic acid (EGTA). Ionic strength, pH corrections and free Ca^{2+} were calculated by a computer program [14, 17]. After incubation at 37° for 5 min, the reactions were terminated by the addition 20 μl 20% trichloroacetic acid (plus 5 mM P_i) and the [^{32}P]-molybdate complex was extracted in isobutanol as described in detail by Ruitenbeek [31]. The drugs were added to the reaction mixture in the form of stock dimethylsulfoxide (DMSO) solutions, until the final medium had a DMSO concentration of 1% (v/v) independent of the drug concentration (controls without drug included). DMSO only slightly (5–10% inhibition) affected enzyme activity.

Assay of PDE. PDE was determined according to a previously reported method [32] using [adenine-^{14}C]-labeled cAMP as a substrate. The reactions were carried out in a medium (0.2 ml) containing 5 mM $MgCl_2$, 100 mM KCl, 0.2 mM $CaCl_2$, 10 mM Tris (pH 7.5, 37°), 37.5 mM mercaptoethanol, 0.25 mM [adenine-U-^{14}C]-cAMP, calmodulin as indicated, and 5 mU/ml of CaM-deficient PDE. The reactions were stopped after 10 min incubation at 37° by adding a 20 μl carrier solution of cAMP, adenosine and AMP each at 30 mM concentration and by immediate placement of the Eppendorf tubes in a 95° thermostat bath for 10 min. The denatured protein was pelleted by centrifugation at 12,000 *g* for 30 sec and spotting 100 μl of the supernatant on Whatmann 3 MM paper. [^{14}C]-AMP was separated from unreacted [^{14}C]-cAMP after an overnight descending chromatography with an elution fluid composed of 30% 1 M ammonium acetate and 70% ethanol (v/v). The spots were visualized under u.v. light and paperstrips containing radioactive AMP were directly counted by liquid scintillation technique in 10 ml Lumagel (Pack-

ard). In some control tests, the Tri-Carb Sample Oxidizer (Packard) was used to completely destroy the paperstrips before solubilization of the residue in the Lumagel. In these studies it was demonstrated that counting of the intact paperstrips is sufficient for estimating the adherent radioactivity.

RESULTS

Effects of felodipine and nifedipine

The inhibition of SL Ca^{2+} pumping ATPase by felodipine is shown in Fig. 1. To determine whether dihydropyridine inhibition is competitive with Ca^{2+}, we also examined the effect of increasing Ca^{2+} concentration on felodipine-induced inhibition. Felodipine was tested at 20 and 50 μM concentrations. Increasing the Ca^{2+} concentration up to 100 μM, which caused a decrease in ATPase activity, slightly modified the degree of inhibition of the Ca^{2+} pumping ATPase by felodipine (Fig. 1). Thus, felodipine at Ca^{2+} levels between 0 and 12 μM does not compete with the binding of Ca^{2+} to the membrane-bound enzyme. This is an unexpected finding, in view of the previously reported data on the potent anti-CaM drug calmidazolium [14, 27, 33]. Calmidazolium, tested at 20 μM concentration, exerted an inhibition of the SL Ca^{2+} pumping ATPase which was competitive with respective to Ca^{2+} ions [14]. It should be noted that calmidazolium [cf. refs. 14 and 33] as well as felodipine had a slightly inhibitory effect on the basal ATPase. This result supports the cautionary observations of other investigators [33–36] that calmidazolium as well as other putative CaM antagonists may directly inhibit enzyme catalysis, independently of any effects on Ca^{2+} sites or Ca^{2+}-calmodulin. The different nature of inhibition by felodipine and calmidazolium on the Ca^{2+} activation of SL Ca^{2+} pumping ATPase suggested that their interaction with soluble CaM-activated PDE might also be distinct. This was further tested by examining the ability of CaM (20–100 nM) to alter drug-induced inhibition of CaM-activated PDE.

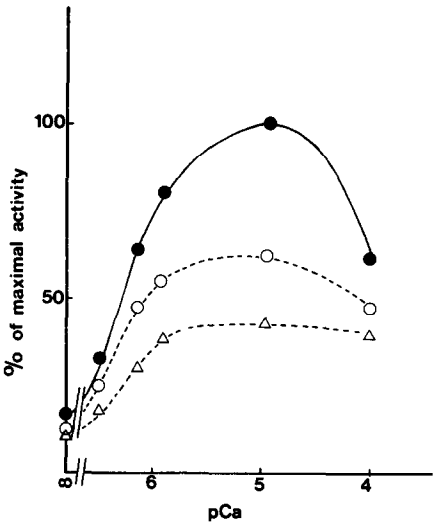

Fig. 1. Inhibition of cardiac SL Ca^{2+} pumping ATPase by felodipine. Ca^{2+} activation curves of SL Ca^{2+} pump are constructed from measurements in the absence of (●) and presence of 20 μM (○) and 50 μM (△) felodipine. ATPase activity was assayed as described in Materials and Methods in the presence of two drug concentrations. Each point represents the mean of at least two determinations. ATPase activity is expressed as percent of the maximum activity, which is measured at 12.2 μM Ca^{2+} free (pCa 4.92)

Because Minocherhomjee and Roufogalis [37] recently reported that the structural analog of felodipine, nifedipine, also depressed CaM-activated PDE without describing effects of increasing CaM concentrations, this drug was also included in the present study. Whereas nifedipine in a similar concentration range of 0–10 μM had only a slightly inhibitory effect, felodipine markedly reduced CaM-activated PDE (Fig. 2). Increasing the CaM concentration up to 100 nM caused no decrease in

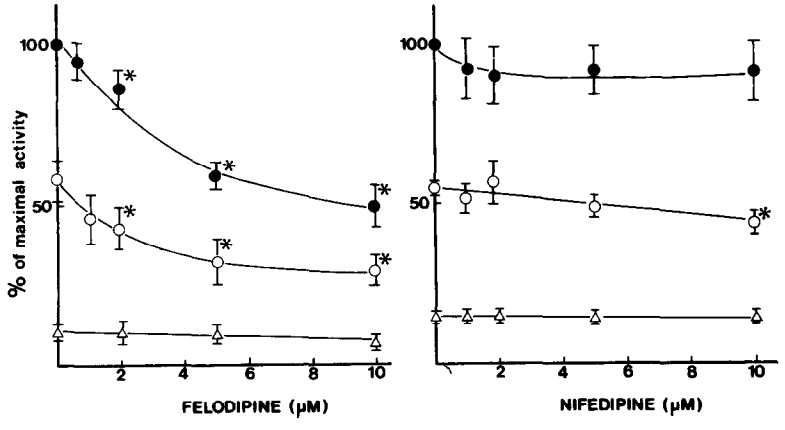

Fig. 2. Effect of slow Ca^{2+} channel blockers on soluble CaM-activated cAMP-PDE purified from dog brain. PDE, either activated with 0 (△), 20 (○) or 100 (●) nM CaM, was assayed as described in Materials and Methods in the presence of various drug concentrations. PDE activity is expressed as percent of the maximum activity, which is measured at 100 nM CaM. Points different from the control with a P value <0.05 are indicated with an asterisk. Each point represents the mean (± S.E.M.) of four experiments.

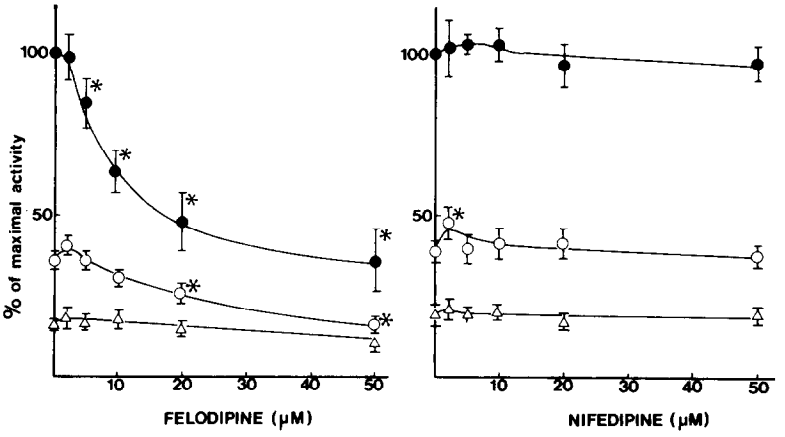

Fig. 3. Effect of slow Ca^{2+} channel blockers on cardiac SL Ca^{2+} pumping ATPase. ATPase, activated with either 0 (△), 0.29 (○) or 12.2 (●) μM Ca^{2+} free concentration, was assayed as described in Materials and Methods in the presence of various drug concentrations. ATPase activity is expressed as percent of the maximum activity, which is measured at 12.2 μM Ca^{2+} free concentration under control conditions. Points different from the control with a P value <0.05 are indicated with an asterisk. Each point represents the mean (± S.E.M.) of four and five experiments (felodipine and nifedipine, respectively).

felodipine-induced inhibition (Fig. 2) suggesting that felodipine does not compete with CaM binding to PDE. This should be compared with the general property of a number of very potent anti-CaM drugs, calmidazolium, trifluoperazin, W-7 and 48/80, to bind the Ca^{2+}-exposed hydrophobic site on CaM which prevents the binding of the Ca^{2+}-CaM complex to and the subsequent activation of PDE [5, 7, 11, 33, 35]. Some of these characteristic CaM inhibitors (calmidazolium and trifluoperazin) have previously been reported to inhibit SL Ca^{2+} pumping ATPase in competition with Ca^{2+} ions [14, 17, 18, 29]. Therefore, it was also suggested that the CaM subunit, endogenously present in the SL Ca^{2+} pumping ATPase preparation, contains, in fact, the Ca^{2+} activation site of the catalysis of ATP hydrolysis [14, 18, 19, 32]. The results shown in Figs. 1 and 2 suggest that felodipine interacts with CaM-activated enzymes in a different manner as the putative CaM antagonists. Previously, stimulatory effects of dihydropyridine Ca^{2+} antagonists on the SL [21] and SR [22, 23, 38] Ca^{2+} pumping ATPase even have been described by testing a wider range of drug concentration (10^{-7}–10^{-5} M). Therefore, in the present work the effects of felodipine and nifedipine were examined on the SL Ca^{2+} pumping ATPase at 2, 5, 10, 20 and 50 μM concentration. The results are illustrated in Fig. 3. It should be noted that in concentrations in which nifedipine did not inhibit the SL Ca^{2+} pumping ATPase felodipine markedly reduced the activity (see also Fig. 1). Furthermore, a slightly stimulatory action of nifedipine and felodipine was seen at 2 μM, although it is very unlikely that this small effect of the dihydropyridine drug has any significance for its pharmacological action. Additional tests with concentrations of the drug

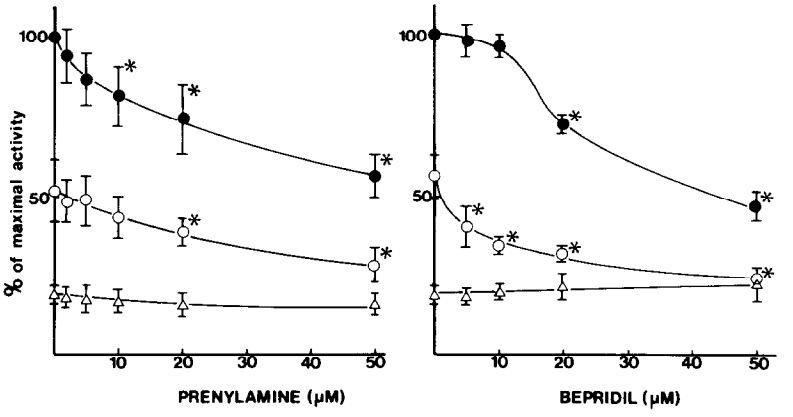

Fig. 4. Effect of slow Ca^{2+} channel blockers on soluble CaM-activated cAMP-PDE purified from dog brain. PDE activities at the various drug concentrations are illustrated as explained for Fig. 2. Points different from the control with a P value <0.05 are indicated with an asterisk. Points represent the mean (± S.E.M.) of seven and six experiments (prenylamine and bepridil, respectively).

lower than 2 μM showed no extra stimulatory effect on the SL Ca^{2+} pumping ATPase (results not shown), which does not agree with the results reported by David-Dulfilho *et al.* [21].

Effects of prenylamine and bepridil

Both prenylamine and bepridil belong to a group of Ca^{2+} slow channel blockers which are known to interact with the intracellular Ca^{2+} receptor CaM and to inhibit the CaM-dependent activation of Ca^{2+}-CaM-dependent enzymes [7, 13, 39, 40]. In Fig. 4 the concentration dependence of prenylamine inhibition of soluble PDE is compared to that of bepridil at two different CaM concentrations (20 and 100 nM). Bepridil displayed a competitive inhibition with respect to CaM at variance with the noncompetitive effect of prenylamine. Prenylamine appears to be a less potent inhibitor than bepridil at 20 nM CaM. The present finding of competitive action of bepridil with CaM confirms previously reported data [39, 40]. Moreover, the sigmoid shape of the bepridil inhibition curve (Fig. 4) may suggest the existence of more than one binding site of the drug on calmodulin having different affinities. The different nature of interaction of prenylamine and bepridil with PDE made it useful to explore possible differences in interaction of the drugs with the SL Ca^{2+} pumping ATPase. As illustrated in Fig. 5, prenylamine (50 μM) is a very poor inhibitor of the Ca^{2+} activated part of the ATPase, whereas bepridil (50 μM) markedly reduced the Ca^{2+} activated part of the ATPase activity at non-saturating Ca^{2+} concentrations (0.29 μM). On the basis of the previous observations with the drug calmidazolium, which also inhibited CaM-activated PDE competitively with CaM, it is not surprising that bepridil inhibition on the Ca^{2+} pumping ATPase is relieved by increasing Ca^{2+} to 12.2 μM [14, 32]. It is rather striking to see even a moderate stimulation of the ATPase by bepridil at a high Ca^{2+} concentration (Fig. 5). Except for the earlier mentioned data on nifedipine [21], the data illustrated in Fig. 5 represent a first example of a Ca^{2+} antagonist which stimulates SL Ca^{2+} pumping ATPase.

DISCUSSION

Our studies demonstrate the ability of two slow Ca^{2+} channel blockers, felodipine and bepridil to inhibit the Ca^{2+} pumping ATPase of cardiac SL, which membrane-bound enzyme recently has been recognized to be CaM-regulated [14, 17, 19, 32]. The inhibition occurs most likely via binding to the CaM subunit of the ATPase as the CaM-dependent activation of the soluble enzyme cAMP-PDE became also inhibited at similar concentrations (Figs. 2 and 4 and compare previous reports [refs. 8, 10, 13, 37, 41]). Furthermore, felodipine, prenylamine and bepridil bind to CaM as shown with a wide variety of techniques: displacement of [^{3}H]-W-7 from CaM [8, 40], increase in fluorescence due to Ca^{2+}-dependent complex formation between *n*-phenyl-1-naphtyl amine and CaM [8, 13], fluorescence increase of dansylated CaM in the presence of Ca^{2+} [5, 7, 26], [^{125}I]-CaM binding to erythrocyte membranes [39] and [^{113}Cd]-NMR spectra of $(Cd)_4$-CaM [3]. The use of the hydrophobic fluorescence probes has shown that when Ca^{2+} binds to its high affinity sites of CaM, it induces a conformational change which exposes hydrophobic regions in the CaM protein. Slow Ca^{2+} channel blockers and the known CaM antagonists (trifluoperazine, calmidazolium and W-7) bind to these hydrophobic sites which has offered an explanation for the Ca^{2+} dependence of drug binding. Drug (e.g. calmidazolium and diltiazem) binding sites on CaM emerged from other studies that could allosterically affect the binding of felodipine at a Ca^{2+}-induced hydrophobic site [5, 26]. Studies using fluorescence probes of displacement of [^{3}H]-trifluoperazine and [^{3}H]-W-7 from CaM pro-

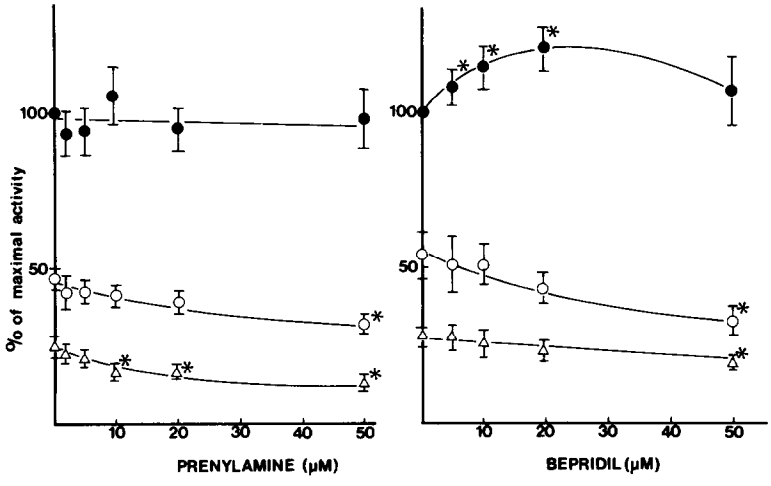

Fig. 5. Effect of slow Ca^{2+} channel blockers on cardiac SL Ca^{2+} pumping ATPase. ATPase activity at the various drug concentrations are illustrated as explained for Fig. 3. Points different from the control with a P value <0.05 are indicated with an asterisk. Each point represents the mean (± S.E.M.) of five and nine experiments (prenylamine and bepridil, respectively).

vided evidence that binding sites of felodipine and prenylamine on CaM are different from those of the potent anti-CaM drugs trifluoperazine, calmidazolium and W-7 [5, 8, 13, 26], whereas bepridil binds to a common site [8, 40]. Indeed, the present study demonstrates that only bepridil inhibition of cAMP-PDE is competitive with CaM as previously found for calmidazolium [14]. That prenylamine was no inhibitor of the Ca^{2+}-activated part of the Ca^{2+} pumping ATPase, is not in agreement with its CaM binding potency [8, 13] and its ability to inhibit cAMP-PDE (Fig. 4). While none of the present experiments conclusively demonstrates inhibition by direct interaction of slow Ca^{2+} channel blockers with CaM, when taken together, the data strongly suggest that the interaction between felodipine (or prenylamine) and CaM is different from the interaction between bepridil and CaM.

Selective binding and subsequent inactivation of CaM action may also result from differences in the CaM environment as some CaM-activated enzymes (cardiac SL Ca^{2+} pumping ATPase and phosphorylase *b* kinase) have previously been shown to possess a CaM subunit which remains bound to the microsomal membrane vesicles, respectively, supernatant fractions after isolation [14–17]. The cardiac SL Ca^{2+} pumping ATPase, described in the present report, may therefore be useful for identification and evaluation of other types of selectivity in anti-CaM action. It should be noted that the cardiac SL Ca^{2+} pumping ATPase has some properties that sets it apart from the more extensively studied plasma-membrane Ca^{2+} pumping ATPase of the erythrocyte: (1) CaM is bound more tightly to the cardiac enzyme [18]; (2) a complex regulatory system mediated by cAMP- and Ca^{2+}-CaM-activated protein kinases only is operative in cardiac SL membranes [reviewed in 42]. *In vitro* the latter protein phosphorylation system can be activated by addition of cAMP or Ca^{2+}-CaM to the membranes, which ingredients were both absent in the present experiments [14, 17, 30, 32, 42]. Apparently CaM required for the action of the intrinsic protein kinase remains not bound to the SL vesicles after isolation at variance with the CaM operative in the direct Ca^{2+} sensitivity regulation of the intrinsic Ca^{2+} pumping ATPase. Previously we demonstrated that calmidazolium interacts with the SL Ca^{2+} pumping ATPase competitively with respect to Ca^{2+} [14]. The present study shows that felodipine inhibits the SL Ca^{2+} pumping ATPase noncompetitively with respect to Ca^{2+} in concentrations not far from those effective in inhibiting cAMP-PDE. That the effective concentration of felodipine in depressing SL Ca^{2+} pumping ATPase is higher than that against cAMP-PDE is not surprising in view of our earlier observations with calmidazolium [14]. The SL membrane protein concentration used in the assay of Ca^{2+} ATPase critically determined the inhibitory potency of calmidazolium. Apparently, the lowered potency to inhibit the SL Ca^{2+} pumping ATPase is due to dilution of the lipophilic drug into the SL lipid bilayer. In conclusion, felodipine and prenylamine possess specific anti-CaM properties which do not resemble those of calmidazolium because: (a) no CaM antagonism of drug action on soluble cAMP-PDE, (b) no Ca^{2+} antagonism of drug action on membrane-bound heart SL Ca^{2+} pumping ATPase, except for prenylamine which was unable to inhibit.

On the basis of the previously reported data [14] and present results illustrated in Fig. 4 a competitive interaction with respect to Ca^{2+} of bepridil with the SL Ca^{2+} pumping ATPase is expected. Our bepridil studies rather indicate that the slight inhibition of suboptimal Ca^{2+} (0.29 μM) can be reversed into a stimulation at higher Ca^{2+} concentration (12.2 μM). Further studies are needed to determine more precisely the relation between Ca^{2+} concentration and bepridil interaction with heart SL Ca^{2+} pumping ATPase. The significance of these observations is that bepridil interacts with CaM like calmidazolium, trifluoperazine and W-7, but that in addition to its inhibiting effect there may be an interaction with CaM or directly with the ATPase, resulting into a stimulation of the enzyme. One restriction should be made to the latter interpretation. Previously we demonstrated that by Ca^{2+} pumping of the vesicle ATPase an electrochemical Ca^{2+} gradient may subsequently inhibit Ca^{2+} pumping ATPase. Bepridil might have uncoupled the uptake process from ATP hydrolysis by increasing vesicle Ca^{2+} permeability [43]. However, reported inhibition by 5–10 μM bepridil of contraction intact smooth muscle makes it rather difficult to believe that bepridil would increase membrane Ca^{2+} permeability [40, 44, 45]. Bepridil (10 μM) had a small inhibitory effect on Ca^{2+} binding to isolated sarcolemma, which result also is rather unexpected if the drug would have increased SL Ca^{2+} permeability [cf. ref. 46]

In view of the observed stimulation of SL ATPase it is interesting that Agre *et al.* [39] reported that bepridil inhibited the erythrocyte Ca^{2+} pumping ATPase, but competitive studies were restricted to CaM. Another important aspect is whether stimulation of the Ca^{2+} pumping ATPase, as observed in cardiac SL vesicles, is reflected by an increase of Ca^{2+} uptake activity. However, proper interpretation of such results will be difficult because of the abundant presence of intact rightside-out vesicles and leaky inside-out vesicles produce an extremely low coupling ratio of transported Ca^{2+} over hydrolyzed ATP [17, 29].

The effects on SL Ca^{2+} pumping ATPase, may provide a model for understanding the interaction of various Ca^{2+} antagonists with membrane proteins which mediate active Ca^{2+} extrusion from the myocardial cell. It also provides additional information on the selectivity in CaM inhibition, when one wants to compare different types of CaM-activated enzymes. It should be stressed, however, that the described effects of felodipine are first seen at concentrations far above those which are therapeutically interesting. Since the structural analog nifedipine in μM amounts had almost no effect on the SL Ca^{2+} pumping ATPase and cAMP-PDE and 1,4 dihydropyridines exert their vasodilator action in the nanomolar range, it seems not plausible that this class of drugs act in the therapeutical ranges through inhibition of CaM activated enzymes [19, 24, 37, 41]. On the other hand, recent reports demonstrate that bepridil on concentrations between 10^{-6} and 10^{-4} M depressed contractions of intact [40, 44–45] as well

as skinned smooth muscle fibers [40, 45]. This is within the range presently shown to be effective in interacting with CaM-activated enzyme systems. Therefore the vasodilatory action of bepridil in intact smooth muscle may be partially dependent on its stimulatory effect on SL Ca^{2+} pumping ATPase and inhibitory action on the MLCK [40]. This is even more likely if one takes into account the large accumulation of bepridil that occurs in muscle in comparison with other slow Ca^{2+} channel blocking drugs, e.g. nifedipine, diltiazem and verapamil [cf. refs. 47 and 48]. Bkaily *et al.* [49] reported recently that trifluoperazine injection into chick embryo cells blocked Ca^{2+} slow inward current. This supports the work of Johnson and Wittenauer [7] which led them to postulate of the existence of a CaM-like protein that controls the Ca^{2+} dependent Ca^{2+} channel closing. Both studies including the present investigation indicate that slow Ca^{2+} channel blockers may exert their action on the Ca^{2+} channel as well as the Ca^{2+} pumping ATPase by affecting their CaM regulation.

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