Bepridil and Cetiedil

Vasodilators which Inhibit Ca^{2+}-dependent Calmodulin Interactions with Erythrocyte Membranes

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Abstract. Two new vascular smooth muscle relaxants, bepridil and cetiedil, were found to possess specific CaM-inhibitory properties which resembled those of trifluoperazine. Trifluoperazine, bepridil, and cetiedil inhibited Ca^{2+}-dependent ^{125}I-CaM binding to erythrocyte membranes and CaM activation of membrane Ca^{2+}-ATPase with IC_{50} values of ~12, ~17, and ~40 μM, respectively. This does not appear to be the result of a nonspecific hydrophobic interaction since inhibition was not observed with micromolar concentrations of many other hydrophobic agents. The predominant inhibition of binding and Ca^{2+}-ATPase activation was competitive with respect to CaM. Bepridil and cetiedil bind directly to CaM since these drugs displaced [^{3}H]trifluoperazine from sites on CaM. Inhibition of Ca^{2+}-ATPase and binding by the drugs was not due to interference with the catalytic activity of this enzyme since: (*a*) neither inhibition of CaM-independent basal Ca^{2+}-ATPase activity nor inhibition of proteolytically-activated Ca^{2+}-ATPase activities were produced by these agents, and (*b*) no drug-induced inhibition of CaM binding was detected when membranes were preincubated with these agents but washed prior to addition of ^{125}I-CaM.

Thus, bepridil and cetiedil competitively inhibit Ca^{2+}-dependent interactions of CaM with erythrocyte membranes, most likely by a direct interaction between these drugs and CaM. The principal clinical actions of these drugs may be explained by their interactions with CaM or CaM-related proteins leading to reduced activation of Ca^{2+}-regulated enzymes in certain other tissues, such as myosin light chain kinase in vascular smooth muscle.

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Introduction

Calmodulin (CaM)[1] is a ubiquitous 17,000 M_r intracellular Ca^{2+}-binding protein known to activate many Ca^{2+}-dependent enzymes and thereby plays a fundamental role in regulation of many physiological events (see monograph, 1, and reviews 2–5). All tissues contain large amounts of CaM (~10^{-6} M) (6), and the list of known CaM-regulated processes is no doubt far from complete. Trifluoperazine and other phenothiazines inactivate CaM (7) by binding to specific sites on CaM in a Ca^{2+}-dependent manner (8). It has been argued that these represent nonspecific hydrophobic interactions rather than true pharmacologic antagonism (9, 10). However, several neuroleptic drugs and other classes of drugs have been shown to bind directly to CaM and specifically antagonize its effects (11, 12), and relationships between drug structure and inhibition have been established (13, 14). No clinically employed drug is presently known to exert its principal pharmacologically desired effects by binding to CaM, although this is an active area of investigation (15).

Clinical medicine is presently experiencing an explosion of interest in use of Ca^{2+} antagonists for the treatment of cardiovascular diseases (see monograph 16, and review 17). Two classes of Ca^{2+} channel blocking drugs are currently used in clinical practice in the United States: "use-dependent" agents (verapamil and diltiazem) and direct channel "plugs" (nifedipine). Two vascular smooth muscle relaxants, bepridil and cetiedil, are currently used in Europe to treat angina and claudication, and these drugs produce some effects similar to the Ca^{2+}-channel blocking agents. Some differences between

1. *Abbreviations used in this paper:* CaM, calmodulin; ^{125}I-CaM, ^{125}I-Bolton Hunter reagent-labeled erythrocyte calmodulin.

the recognized Ca^{2+}-channel blockers and bepridil and cetiedil include: (*a*) much higher concentrations of bepridil and cetiedil are required, (*b*) the electrophysiologic effects are distinct, and (*c*) bepridil and cetiedil both produce effects on a wide range of other tissues. Despite investigation of bepridil and cetiedil in several laboratories, the biochemical mechanisms of their actions are unknown.

This report describes the use of a recently developed erythrocyte system for identification and evaluation of potential CaM-inhibitors (18, 19). It was found that bepridil and cetiedil competitively inhibit Ca^{2+}-dependent CaM binding to membrane receptor sites and displace trifluoperazine from sites on CaM, probably by directly interacting with CaM. The principally desired pharmacologic effect, relaxation of vascular smooth muscle, and possibly effects upon other tissues are probably sequelae of inhibition of CaM-dependent enzymes.

Methods

Reagents. Bepridil, β-(2-methylpropoxy)methyl-*N*-phenyl-*N*-(phenylmethyl)-1-pyrrolidineethanamine monohydrochloride monohydrate and cetiedil, 2-(hexahydro-1H-azepin-1-yl)ethyl α-cyclohexyl-3-thiophenacetate 2-hydroxy-1,2,3-propanetricarboxylate hydrate (1:1:1) were provided by McNeil Pharmaceutical (Spring House, PA). Other drugs from a variety of industrial sources were provided by Dr. Robert Gould, Department of Neurosciences, Johns Hopkins Medical School. ^{125}I-Bolton Hunter reagent was from Amersham Corp., Arlington Heights, IL; (γ-^{32}P)ATP (~25 Ci/mmol) was from ICN Radiochemicals, Irvine, CA; [^{3}H]trifluoperazine (72 Ci/mmol) was from New England Nuclear, Boston, MA; α-chymotrypsin (54 u./mg) was from Millipore, Bedford, MA; ultra pure sucrose was from Schwarz/Mann; NaEGTA, ouabain, trifluoperazine, phenylmethylsulfonyl fluoride, dithiothreitol, and Norit A were from Sigma Chemical Co., St. Louis, MO; Hepes was from Research Organics, Inc., Cleveland, OH; and other reagent grade chemicals were used. Pure erythrocyte CaM was prepared and radiolabeled to ~70,000 cpm/pmol with ^{125}I-Bolton Hunter reagent as described (19).

Membrane preparations. Blood was obtained from normal adult volunteers by venipuncture, anticoagulated with acid citrate dextrose, and stored for up to 3 d at 0°C. Erythrocyte ghost membranes were prepared from erythrocytes washed three times in 0.15 M NaCl, 5 mM $NaPO_4$, pH 7.4, and lysed in 40 vol of ice-cold 7.5 mM $NaPO_4$, 1 mM NaEGTA, 35 μg/ml phenylmethylsulfonyl fluoride, pH 7.3, centrifuged at 44,000 *g* for 10 min and washed repetitively until white. The membranes were then washed once in 10 mM Hepes, pH 7.3, and stored at 0°C in 10 mM Hepes, pH 7.3, 0.1 mM dithiothreitol, 1 mM NaN_3 for up to 1 d. Spectrin-actin stripped membrane vesicles were prepared by incubating fresh ghost membranes in 40 vol of 0.3 mM $NaPO_4$, pH 7.4, at 37°C for 30 min. The vesicles were pelleted at 44,000 *g* for 25 min, washed again in 10 mM Hepes, pH 7.3, and resuspended to 1.5 mg protein/ml in 10 mM Hepes, 0.1 mM dithiothreitol, 1 mM NaN_3. α-chymotrypsin-digested vesicles were prepared basically as described (19). Spectrin-actin stripped membrane vesicles (1.5 mg protein/ml) were incubated for 45 min at 0°C with 8 μg/ml α-chymotrypsin (diluted from a fresh stock solution of 4 mg α-chymotrypsin in 1 ml of 2 mM HCl), then washed twice in ice cold 10 mM Hepes (pH 7.3) also containing 50 μg/ml phenylmethylsulfonyl fluoride, and resuspended to 1.2 mg protein/ml in 10 mM Hepes, 0.1 mM dithiothreitol, 1 mM NaN_3.

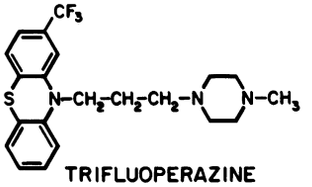
Binding assay. Inhibition of Ca^{2+}-dependent ^{125}I-Bolton Hunter reagent-labeled erythrocyte calmodulin (^{125}I-CaM) binding to erythrocyte ghost membranes was measured as described (19). Glass tubes were employed due to adherence of some drugs to plastic. Generally trifluoperazine, bepridil, cetiedil, or other drugs were freshly dissolved to ~0.5 mM in distilled water, and dilutions were added to ^{125}I-CaM (0.3–10 nM diluted in 0.25 mg/ml gelatin) in 80 mM NaCl, 30 mM KCl, 0.5 mM $MgCl_2$, 30 mM Hepes (pH 7.3) either with buffered Ca^{2+} (2.50 mM $CaCl_2$/2.50 mM NaEGTA, pCa^{2+} = 5.0) or chelator (2.50 mM NaEGTA, $pCa^{2+} > 8$). Erythrocyte ghost membranes were added last to a final volume of 0.2 ml, and incubations were performed at room temperature until binding equilibrium was established (4–5 h). Specific membrane binding was determined by layering 0.18-ml aliquots over a small airspace above 0.2 ml of the same buffer containing 20% (wt/vol) sucrose in hard polyethylene Eppendorf microtest tubes. The tubes were spun at 35,000 *g* for 30 min and frozen in crushed dry ice. The tips (containing ^{125}I-CaM bound to membranes) were clipped off and assayed for ^{125}I in a gamma counter. The tops (containing unbound ^{125}I-CaM) were likewise assayed. Ca^{2+}-dependent membrane binding was determined by subtracting counts per minute from tips of parallel incubations under identical conditions wherein NaEGTA was included without $CaCl_2$. Duplicate values in the binding assay were within 5%.

Ca^{2+}-ATPase assay. Analysis of Ca^{2+}-dependent ATP hydrolysis was measured basically as described (19). Freshly dissolved drugs, unlabeled pure erythrocyte CaM, and erythrocyte membranes were incubated exactly as described for the binding assay also including 0.1 mM ouabain. After binding equilibrium was established, (γ-^{32}P)ATP was added for 30 min at room temperature and the reaction was stopped during the linear stage of ATP hydrolysis by addition of 1.0 ml of ice-cold 10% (wt/vol) trichloroacetic acid followed by 0.5 ml of 10% (wt/vol) Norit A. The tubes were centrifuged 3000 *g* for 10 min and 1.0-ml aliquots of the supernatant (free P_i) were assayed for ^{32}P in a liquid scintillation counter after addition of 5 ml Beckman Ready-Solv (Beckman Instruments, Inc., Fullerton, CA). Background P_i levels were subtracted (counts per minute measured without Ca^{2+} or without membranes). Duplicate determinations were within 5%.

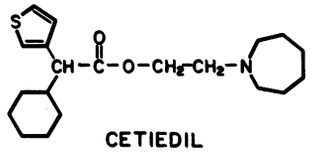
Results

Bepridil and cetiedil inhibit Ca^{2+}-dependent CaM-binding to membranes. CaM associations with erythrocyte membranes have been analyzed with a binding assay employing ^{125}I-labeled CaM (18, 19). The Ca^{2+}-transporter (Ca^{2+}-ATPase) is a principal high affinity CaM binding site on the membrane ($K_d < 10^{-9}$ M), and associations with additional membrane sites are also measured with this probe (19). This relatively simple and highly reproducible assay will permit rapid analysis of many different agents for ability to inhibit ^{125}I-CaM binding to these sites. Effects of drugs upon unlabeled CaM may be evaluated by measurement of Ca^{2+}-ATPase activation. Erythrocyte Ca^{2+}-ATPase has been purified and well-defined biochemically (20). A similar enzyme is present in other tissues (21), and therefore, results with erythrocytes are likely to have more general significance. Phenothiazines (22) and certain classes of experimental drugs (23) inhibit a variety of CaM-dependent enzymes by directly interacting with CaM. Trifluoperazine at $\sim 10^{-4}$ M was found both to inhibit and reverse Ca^{2+}-dependent ^{125}I-CaM binding to sites on erythrocyte membranes (19). Many

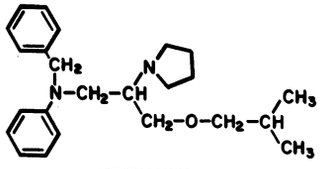
different hydrophobic drugs from several therapeutic classes were evaluated for ability to inhibit ^{125}I-CaM binding. Negligible inhibition of 10 nM ^{125}I-CaM binding was observed with 100 μM concentrations of the following drugs: nifedipine, neoverapamil, diltiazem, papaverine, quinidine, procainamide, lidocaine, phenytoin, ouabain, atropine, phentolamine, propranolol, yohimbine, reserpine, isoproterenol, arecoline, aminophylline, dibucaine, doxepin, mesoridazine, diazepam, spiperone, diphenhydramine, cimetidine, colchicine, indomethacin, chloroquine, cinchonine, berberine, cytochalasin, boldine, and aconitine. However, two vascular smooth muscle relaxants, bepridil and cetiedil (Fig. 1), were found to be potent antagonists of Ca^{2+}-dependent ^{125}I-CaM binding. Both drugs at 50 μM reduced binding of 10 nM ^{125}I-CaM by >50%, whereas many other hydrophobic drugs (including several Ca^{2+} channel blockers) failed to interfere significantly with ^{125}I-CaM binding. Reduction in Ca^{2+}-dependent ^{125}I-CaM binding produced by 50 μM bepridil and cetiedil do not result from reductions in free Ca^{2+} concentrations due to chelation of Ca^{2+} by these drugs, since 2.50 mM $CaCl_2$/2.50 mM EGTA will buffer against such effects. Bepridil and cetiedil are known to block several Ca^{2+}-dependent physiologic effects in a variety of tissues. Furthermore, these compounds have structural features previously identified as important for binding to CaM (13): a hydrophobic aromatic ring system with a flexible side chain containing an amino group which should be positively charged at physiologic pH. It was considered possible that inhibition of CaM may be the basis for some of these effects, so specific actions of bepridil and cetiedil upon CaM binding to erythrocyte membranes and activation of Ca^{2+}-ATPase were therefore explored.



TRIFLUOPERAZINE



CETIEDIL



BEPRIDIL

Figure 1. Structures of trifluoperazine, cetiedil, and bepridil.

Inhibition of binding and Ca^{2+}-ATPase is predominantly competitive with respect to CaM. Inhibition of Ca^{2+}-dependent CaM-binding may result from direct interaction of drugs with CaM (leading to inactivation of CaM) or by reversible interactions of drugs directly with CaM binding sites on the membrane. Both types of interactions should be competitive with respect to CaM. Inhibition may also result from interaction of drugs with other sites on the membrane, which create secondary effects upon the CaM binding sites, or by irreversible damage to the binding site (noncompetitive inhibition). To distinguish competitive from noncompetitive interactions, inhibition of ^{125}I-CaM binding and activation of Ca^{2+}-ATPase were measured at multiple CaM concentrations. Binding of 0.3, 0.8, and 2 nM ^{125}I-CaM was inhibited by trifluoperazine, bepridil, and cetiedil with inhibitory concentration of 50% (IC_{50}) values of 10–12, 14–19, and 32–39 μM, respectively (Fig. 2). Competitive inhibition is best analyzed with Dixon plots (24) wherein the reciprocals of enzyme activation (or binding) at two or more concentrations of a specific ligand are plotted against increasing concentrations of inhibitor. In simple bimolecular interactions, the plots are linear and extrapolations intersect at inhibitor concentrations corresponding to negative inhibition constant (K_I). Precise determinations of the K_I were not possible in these experiments since Dixon plots (not shown) were parabola-shaped at these drug concentrations. Nonlinear Dixon plots suggest that the drug-CaM inhibition may be complex (possibly cooperative). Double reciprocal plots of these data are shown as inserts in Fig. 2. The principal inhibition by all three agents is competitive with respect to CaM. Linear regressions have increasing slopes at increasing drug concentrations, and each clearly projects to a different point on the x axis ($1/K_d$). As expected for a competitive inhibition, the projections intersect the y axis at nearly the same point, the reciprocal of the expected high affinity binding capacity, ~10 pmol/mg membrane protein (19). However, projections at the higher drug concentrations (30 μM trifluoperazine, >30 μM bepridil, and >45 μM cetiedil) intersect the y axis at slightly higher points indicating that secondary noncompetitive interactions may also occur.

Basal and CaM-stimulated Ca^{2+}-ATPase activities were measured in the presence of trifluoperazine, bepridil, and cetiedil (Fig. 3). None of the drugs produced notable reductions in basal Ca^{2+}-ATPase activity at drug concentrations which produced nearly total inhibition of CaM-stimulated activity. At 0.3–2 nM CaM, IC_{50} values were 13–14 μM trifluoperazine, 17–19 μM bepridil, and 39–53 μM cetiedil. Precise K_I determinations at these drug and CaM concentrations were not possible due to nonlinearity of these Dixon plots also (not shown). Double reciprocal plots of these data are shown as inserts in Fig. 3. Enzyme inhibition by all three drugs appears to be competitive with CaM, and the y intercepts approximate the reciprocal of the expected CaM-stimulated Ca^{2+}-ATPase activities, V_{max} = 6–10 nmol P_i/mg membrane protein per minute (19). As was found in the binding studies (Fig. 2),

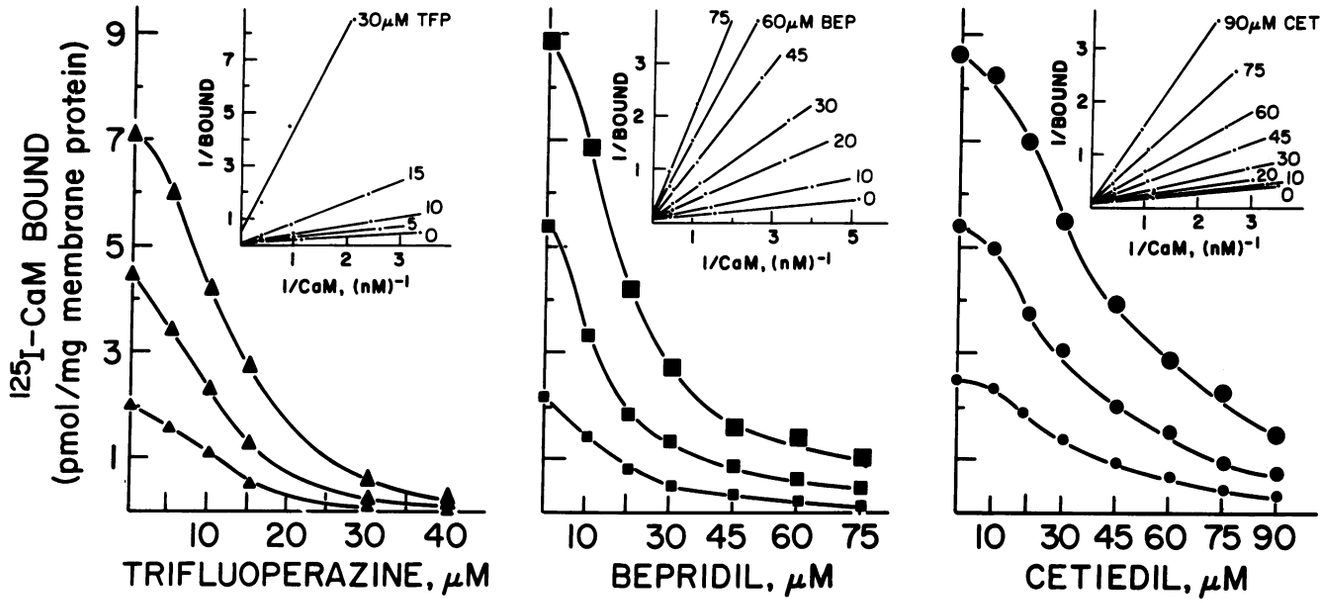


Figure 2. Inhibition of Ca^{2+}-dependent ^{125}I-CaM binding to erythrocyte ghost membranes by increasing concentrations of trifluoperazine, bepridil, and cetiedil. Various concentrations of these agents were incubated with 0.3 nM (▲, ■, ●), 0.8 nM (▲, ■, ●), or 2 nM (▲, ■, ●) ^{125}I-CaM (68,000 cpm/pmol) and erythrocyte ghost membranes (20 μg protein) for 5 h before specific binding was determined (see Methods). The insets contain linear regression analyses of the reciprocals of bound and unbound ^{125}I-CaM measured at each drug concentration.

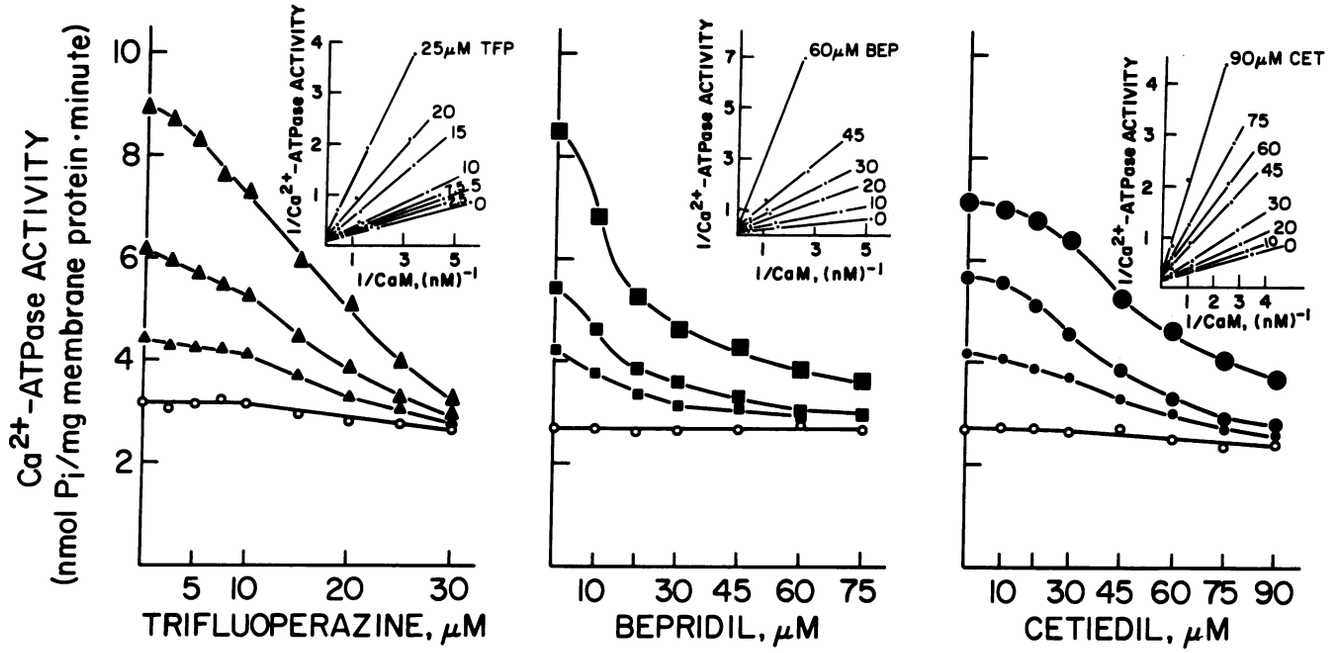


Figure 3. Inhibition of CaM-stimulated erythrocyte ghost membrane Ca^{2+}-ATPase activity by increasing concentrations of trifluoperazine, bepridil, and cetiedil. Various concentrations of these agents were incubated in the absence of CaM (○) or in the presence of 0.3 (▲, ■, ●), 0.8 (▲, ■, ●), or 2 nM CaM (▲, ■, ●) and erythrocyte ghost membranes (20 μg protein) for 5 h before ^{32}P-ATP (10,000–15,000 cpm/nmol) was added to 50 μM and Ca^{2+}-dependent ATP hydrolysis was measured (see Methods). The insets contain linear regression analyses of reciprocals of CaM-activated Ca^{2+}-ATPase activities (basal subtracted) and unbound CaM concentrations (determined by parallel ^{125}I-CaM binding assay, see Fig. 2) measured at each drug concentration.

linear regression of double reciprocal data at higher drug concentrations project to slightly higher y intercepts indicating the existence of secondary, noncompetitive interactions which produce reductions in apparent V_{max}.

As expected for competitive interactions, drug inhibition of CaM-dependent Ca^{2+}-ATPase activity can be overcome by increasing concentrations of CaM. 40 μM trifluoperazine, 80 μM bepridil, and 100 μM cetiedil are at or above the concentrations which produced nearly complete inhibition of CaM binding (Fig. 2) and CaM-activated Ca^{2+}-ATPase (Fig. 3). These drug concentrations were employed in Ca^{2+}-ATPase assays with concentrations of CaM rising from 2 to 128 nM (Fig. 4). In the absence of drug, 2 nM CaM stimulated the enzyme nearly maximally. In the presence of each drug, little CaM stimulation was measured at 2 nM CaM; however, the inhibition was reversed with increasing CaM concentrations and approached V_{max} at 128 nM CaM.

Drugs do not damage membrane target sites for CaM. An important consideration in these studies is to evaluate possible nonspecific effects of these drugs on CaM target sites. The catalytic activity of the Ca^{2+}-ATPase in the absence of CaM is unaffected by these drugs. Basal Ca^{2+}-ATPase activity was only slightly reduced by increasing concentrations of trifluoperazine, bepridil, and cetiedil (Figs. 3 and 5). Direct effects of CaM-inhibitory drugs upon stimulated enzyme catalytic activity cannot be evaluated when CaM is present. However, full Ca^{2+}-ATPase activity can be evoked in the absence of CaM by controlled proteolytic removal of the CaM-binding regulatory domain of the enzyme. The resulting catalytic activity (deregulated activity) is insensitive to CaM, but otherwise closely

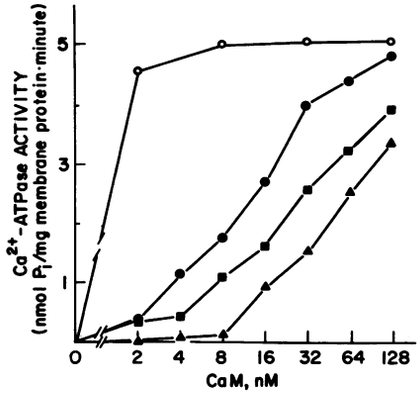


Figure 4. Restoration of CaM-dependent Ca^{2+}-ATPase activity in erythrocyte ghost membranes incubated with trifluoperazine, bepridil, or cetiedil by increasing concentrations of CaM. Erythrocyte ghost membranes (24 μg protein) were incubated with increasing concentrations of CaM in the presence of 40 μM trifluoperazine (▲), 80 μM bepridil (■), 100 μM cetiedil (●), or with no additional drug (○) for 2.5 h before ^{32}P-ATP (10,000 cpm/nmol) was added to 50 μM and Ca^{2+}-dependent ATP hydrolysis was measured (see Methods). Basal Ca^{2+}-ATPase activities were subtracted.

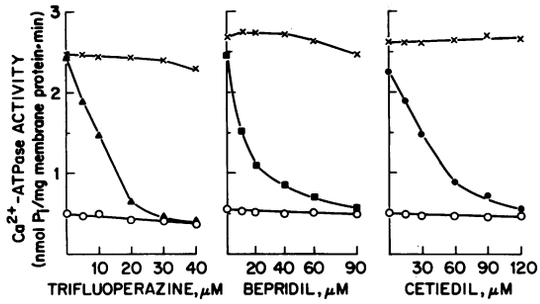


Figure 5. Lack of inhibition of CaM-independent Ca^{2+}-ATPase activities by increasing concentrations of trifluoperazine, bepridil, or cetiedil. Spectrin-stripped membrane vesicles were prepared from fresh erythrocyte ghost membranes, and a portion of these were mildly digested with α-chymotrypsin under controlled conditions (see Methods). Digested membrane vesicles (×; 13 μg protein) or undigested membrane vesicles (○, ▲, ■, ●; 18 μg protein) were incubated with increasing concentrations of trifluoperazine, bepridil, or cetiedil with 2 nM CaM (▲, ■, ●) or without CaM (○, ×) for 3 h before ^{32}P-ATP (16,000 cpm/nmol) was added to 16 μM and Ca^{2+}-dependent ATP hydrolysis was measured (see Methods).

resembles CaM stimulation of the intact enzyme (25). Vesicles with proteolytically stimulated Ca^{2+}-ATPase activity were incubated with increasing concentrations of trifluoperazine, bepridil, and cetiedil (Fig. 5). While this range of drug concentrations nearly totally abolished CaM-stimulated Ca^{2+}-ATPase activity, the proteolytically stimulated enzyme activity was virtually undiminished. Thus, it appears that the principal drug inhibition of Ca^{2+}-ATPase activity is not due to direct interaction of the agents with the catalytic domain of the enzyme.

Lack of identifiable, persistent effects of drugs directly upon membrane binding sites. Preincubation experiments failed to identify drug-membrane interactions leading to reduced ^{125}I-CaM binding if the membranes were washed prior to addition of ^{125}I-CaM. Erythrocyte membranes were incubated in buffered Ca^{2+} in the absence of drug or in the presence of trifluoperazine, bepridil, or cetiedil (Fig. 6). If ^{125}I-CaM was then added, binding was significantly inhibited. If the membranes were preincubated with drugs but washed in Ca^{2+}-containing buffers before addition of ^{125}I-CaM, inhibition of ^{125}I-CaM binding was no longer detected. Thus, it is unlikely that the reduced ^{125}I-CaM binding is due to irreversible-direct Ca^{2+}-dependent interaction of the drugs with the membrane binding sites. It is still conceivable that drug-membrane interactions may occur that are rapidly and completely reversed by washing the membranes in Ca^{2+}-containing buffers.

Bepridil and cetiedil displace [^{3}H]trifluoperazine from sites on CaM. Trifluoperazine has been shown to bind directly to sites on CaM in the presence of Ca^{2+}, and the number of sites and affinity of the interactions have been measured by equilibrium dialysis (8). Similar [^{3}H]trifluoperazine displacement measurements were performed in the presence of increasing

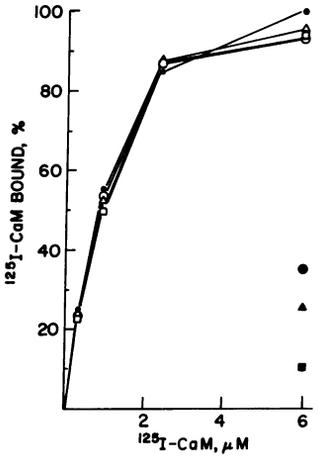


Figure 6. Lack of persistent CaM-binding inhibition after washing erythrocyte membranes previously incubated with trifluoperazine, bepridil, or cetiedil. Erythrocyte ghost membranes (2 mg protein) were incubated for 45 min at room temperature in 6 ml of 80 mM NaCl, 30 mM KCl, 30 mM Hepes (pH 7.3), 2.50 mM $CaCl_2$/2.50 mM NaEGTA (pCa^{2+} = 5.0), with 25 μM trifluoperazine (△, ▲), 60 μM bepridil (□, ■), 80 μM cetiedil (○, ●), or no other addition (•). 0.5-ml aliquots were removed from each tube for analysis of Ca^{2+}-dependent binding of 6 nM ^{125}I-CaM (•, ▲, ■, ●). The remaining membranes (•, △, □, ○) were washed by dilution to 40 ml in the same buffer, pelleted at 44,000 *g* for 15 min, and resuspended in 2 ml of 1 mM Hepes, pH 7.3, immediately before analysis of binding 0.36, 0.96, 2.4, or 6 nM ^{125}I-CaM (see Methods). 100% bound refers to maximum binding of 6 nM ^{125}I-CaM by the membranes in the unwashed and washed groups to which no additional drugs were added during the preincubation. Significant differences are noted between control (•) and drug-preincubated membranes in the unwashed group (▲, ■, ●) while no differences persist between control (•) and drug-preincubated membranes in the group washed before addition of ^{125}I-CaM (△, □, ○).

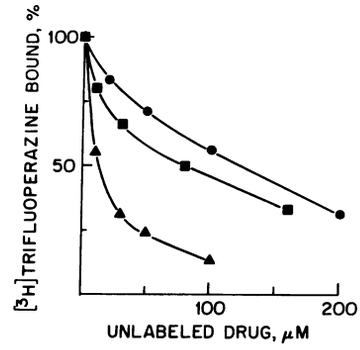


Figure 7. Displacement of [^{3}H]trifluoperazine from sites on CaM by equilibrium dialysis with increasing concentrations of unlabeled trifluoperazine, bepridil, or cetiedil. Dialysis bags (0.5 ml) containing 2.5 μM CaM and 0.5 mg gelatin or 0.5 mg gelatin alone were dialyzed to equilibrium by gently shaking for 15 h at room temperature in the dark in glass beakers containing 20 ml of 0.1 M Hepes, 2.50 mM $CaCl_2$/2.50 mM NaEGTA (pCa = 5.0), 1 mM NaN_3, 10 μM [^{3}H]trifluoperazine (80,000 cpm/ml), and also containing increasing concentrations of unlabeled trifluoperazine (▲), bepridil (■), or cetiedil (●). The bags were snipped and aliquots from the bags containing CaM in gelatin, the bags containing gelatin alone, and dialysis buffer were assayed for ^{3}H in 5 ml of Beckman Ready-Solv. 100% refers to counts per minute specifically bound to CaM (counts per minute in CaM-gelatin bag minus counts per minute in gelatin bag = 22,500 cpm/200 μl) after dialysis to equilibrium against 10 μM [^{3}H]trifluoperazine with no additional unlabeled drug present, as described by Weiss (22).

concentrations of unlabeled trifluoperazine, bepridil, and cetiedil (Fig. 7). All three drugs displace [^{3}H]trifluoperazine with the relative potencies of trifluoperazine ≫ bepridil > cetiedil. The fraction of [^{3}H]trifluoperazine displaced by bepridil and cetiedil was less than anticipated when compared with the potencies with which they inhibited Ca^{2+}-dependent ^{125}I-CaM binding and CaM stimulation of Ca^{2+}-ATPase. The explanation for this is uncertain, but it may be due to interaction of bepridil and cetiedil with sites on CaM which only partially overlap those to which trifluoperazine binds, or due to noncompetitive inhibition of [^{3}H]trifluoperazine binding to CaM as was described for displacement of ^{3}H-W7 by prenylamine in a preliminary report (26). The difference may also possibly result from relatively greater nonspecific adsorption of unlabeled bepridil and cetiedil onto dialysis membranes (resulting in reduction in the actual bepridil or cetiedil concentrations in solution).

Discussion

Trifluoperazine, bepridil, and cetiedil inhibited Ca^{2+}-dependent binding of ^{125}I-CaM to erythrocyte membranes and inhibited CaM activation of membrane Ca^{2+}-ATPase with IC_{50} values of ~12, ~17, and ~40 μM, respectively. Detailed analyses indicate that the inhibition at concentrations of up to 20 μM trifluoperazine, 30 μM bepridil, and 45 μM cetiedil is entirely competitive with respect to CaM. None of the three drugs produced identifiable direct effects upon the membranes: none produced inhibition either of basal Ca^{2+}-ATPase or CaM-independent (proteolytically stimulated) Ca^{2+}-ATPase activity, and none produced reductions in membrane-CaM binding which persisted after membrane washing. Bepridil and cetiedil displaced [^{3}H]trifluoperazine from sites on CaM, although with somewhat less potency than did unlabeled trifluoperazine. Thus, bepridil and cetiedil possess specific CaM-inhibitory properties which resemble those of trifluoperazine in several ways: (*a*) CaM-membrane interactions are competitively inhibited by these agents, (*b*) no effects of drugs acting directly on membranes were identified at these concentrations, and (*c*) the agents displaced trifluoperazine from sites on CaM. While none of the experiments conclusively demonstrated inhibition by direct interaction of bepridil or cetiedil with CaM, when taken together, these data are most consistent with that explanation. However, it is not possible to rule out secondary, reversible interactions of the drug with membrane target sites that result in inhibition of CaM binding. Such studies will require purification of CaM-binding proteins and direct measurements of radiolabeled drugs to these CaM-binding proteins.

Despite active clinical and physiologic interest in the multiple effects of bepridil and cetiedil, the biochemical bases of these effects have not been uncovered. The studies in this report indicate that bepridil and cetiedil may deliver primary effects by interacting with CaM leading to inhibition of CaM-dependent enzymes. Bepridil and cetiedil act pharmacologically and inhibit Ca^{2+}-dependent ^{125}I-CaM binding and CaM-activation of Ca^{2+}-ATPase at similar concentrations. Bepridil and

cetiedil are used clinically in Europe for the treatment of angina and claudication, and several other beneficial cardiovascular effects have been noted in experimental animals (27–32). Although most are preliminary, several recent reports noted very encouraging antianginal (33, 34) and antiarrhythmic results (35–39) in clinical studies with bepridil. Some of these benefits may be derived from inhibition of CaM-regulated myosin light chain kinase in vascular smooth muscle. Other effects may result from bepridil and cetiedil acting directly on cardiac muscle which contains large amounts of CaM in addition to troponin C, a related protein. CaM exists in all eukaryotic tissues, and not surprisingly, micromolar concentrations of bepridil and cetiedil inhibit numerous other Ca^{2+}-dependent processes in a variety of tissues. Bepridil reduces Ca^{2+}-dependent target cell lysis by *Entamoeba histolytica* (40). Cetiedil inhibits Ca^{2+}-induced cation fluxes in erythrocytes (41) and may be of value in sickle cell disease (42–44). Cetiedil was found to inhibit CaM-stimulated brain phosphodiesterase and erythrocyte Ca^{2+}-ATPase (45, 46) and specifically modulates certain Ca^{2+}-dependent polymorphonuclear leukocyte functions (47) and platelet aggregation. While it is likely that some of these effects may result from inhibition of CaM-dependent enzymes or CaM binding proteins, other CaM-inhibitors have been found to inhibit additional Ca^{2+}-sensitive enzymes in the absence of CaM, such as protein kinase C (48–50) and phosphatase IIb (51). Several other proteins bound directly to phenothiazine columns in the presence of Ca^{2+} even though they did not contain CaM subunits and failed to bind to CaM-affinity columns (52). Thus, it is likely that certain effects of bepridil and cetiedil are not due to inhibition of CaM.

Bepridil and cetiedil may belong to a new class of vascular smooth muscle relaxants which achieve their principal pharmacologic effects by inhibition of CaM and other intracellular Ca^{2+}-sensitive processes rather than by direct interaction with the Ca^{2+} channels which regulate Ca^{2+} entry. Therefore, bepridil and cetiedil are different from the recognized Ca^{2+} channel blockers (although some of the latter have also been found to interact with CaM) (53–55). Certain other smooth muscle relaxants with related structures including naphthalenesulfonamides (W7 and No. 233) and prenylamine (another European coronary vasodilator) interact directly with CaM with comparable affinities, inhibit CaM-activated myosin light chain kinase (12), and may also be considered members of this class of drugs. These agents all appear to achieve at least some of their principal pharmacologic effects by inhibition of CaM-dependent enzymes. These agents are also distinct from phenothiazines, which produce their principal antipsychotic effects at very low concentrations by interaction with dopamine receptors but produce side effects at higher concentrations probably due to interaction with CaM.

All tissues contain CaM, and since many CaM-dependent enzymes are essential for cell survival, it appears paradoxical that unselective inhibition of CaM is not deleterious. CaM inhibitors have been demonstrated to inhibit progression in the cell cycle from G1 to DNA-S phase (56). Erythrocyte Ca^{2+}-ATPase is inhibited by phenothiazines (57), and accumulation of intracellular Ca^{2+} can produce toxic activation of intracellular proteases (58) and transglutaminase (59). Therefore, for CaM inhibition to be clinically useful, there must be inhibition of certain enzymes more than others. Selective inhibition may result from differences in drug levels in different tissues or even by enzyme localizations within a cell. It is also likely that the affinity with which CaM activates certain enzymes may be important in selective enzyme inhibition. The system described in this report may be useful for identification and evaluation of selective CaM inhibitors.

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References

1. Cheung, W. Y. 1980. Calmodulin. Calcium and Cell Function. Vol. I. Academic Press, New York. 1–379.

2. Cheung, W. Y. 1980. Calmodulin plays a pivotal role in cellular regulation. *Science (Wash. DC).* 207:19–27.

3. Means, A. R., and J. R. Dedman. 1980. Calmodulin—an intracellular calcium receptor. *Nature (Lond.).* 285:73–77.

4. Klee, C. B., T. H. Crouch, and P. G. Richman. 1980. Calmodulin. *Annu. Rev. Biochem.* 49:489–515.

5. Klee, C. B., and T. C. Vanaman. 1982. Calmodulin. *Adv. Protein Chem.* 35:213–321.

6. Chafouleas, J. G., J. R. Dedman, R. P. Munjaal, and A. R. Means. 1979. Calmodulin: development and appliction of a sensitive radioimmunoassay. *J. Biol. Chem.* 254:10262–10267.

7. Uzunov, P., and B. Weiss. 1971. Effects of phenothiazine tranquilizers on the cyclic 3′,5′-adenosine monophosphate system of rat brain. *Neuropharmacology.* 10:697–708.

8. Levin, R. M., and B. Weiss. 1977. Binding of trifluoperazine to the calcium-dependent activator of cyclic nucleotide phosphodiesterase. *Mol. Pharmacol.* 13:690–697.

9. Norman, J. A., and A. H. Drummond. 1979. Inhibition of calcium-dependent regulator-stimulated phosphodiesterase activity by neuroleptic drugs is unrelated to their clinical efficiency. *Mol. Pharmacol.* 16:1089–1094.

10. Roufogalis, B. D. 1981. Phenothiazine antagonism of calmodulin: a structurally nonspecific interaction. *Biochem. Biophys. Res. Commun.* 98:607–613.

11. Levin, R. M., and B. Weiss. 1979. Selective binding of antipsychotics and other psychoactive agents to the calcium-dependent activator of cyclic nucleotide phosphodiesterase. *J. Pharmacol. Exp. Ther.* 208:454–459.

12. Hidaka, H., T. Yamaki, M. Naka, T. Tanaka, H. Hayashi, and R. Kobayashi. 1980. Calcium-regulated modulator protein interacting

agents inhibit smooth muscle calcium-stimulated protein kinase and ATPase. *Mol. Pharmacol.* 17:66–72.

13. Prozialek, W. C., and B. Weiss. 1982. Inhibition of calmodulin by phenothiazines and related drugs: structure activity relationships. *J. Pharmacol. Exp. Ther.* 222:509–516.

14. Nishikawa, M., and H. Hidaka. 1982. Role of calmodulin in platelet aggregation: structure-activity relationships of calmodulin and antagonists. *J. Clin. Invest.* 69:1348–1355.

15. Weiss, B., W. C. Prozialek, and T. L. Wallace. 1982. Interaction of drugs with calmodulin. Biochemical, pharmacological, and clinical implications. *Biochem. Pharmacol.* 13:2217–2226.

16. Flaim, S. F., and R. Zelis. 1982. Calcium Channel Blockers: Mechanisms of Action and Clinical Applications. Urban and Schwarzenberg, Baltimore. 121–283.

17. Braunwald, E. 1982. Mechanism of action of calcium-channel-blocking agents. *N. Engl. J. Med.* 307:1618–1627.

18. Graf, E., A. G. Filoteo, and J. T. Penniston. 1980. Preparation of ^{125}I-CaM with retention of full biological activity: its binding to human erythrocyte ghost. *Arch. Biochem. Biophys.* 203:719–726.

19. Agre, P., K. Gardner, and V. Bennett. 1983. Association between human erythrocyte calmodulin and the cytoplasmic surface of human erythrocyte membranes. *J. Biol. Chem.* 258:6258–6265.

20. Niggli, V., E. S. Adunyah, J. T. Penniston, and E. Carafoli. 1981. Purified (Ca^{2+} + Mg^{2+}) ATPase of the erythrocyte membrane. Reconstitution and effects of calmodulin and phospholipids. *J. Biol. Chem.* 256:395–401.

21. Caroni, P., and E. Carafoli. 1981. The Ca^{2+} pumping ATPase of heart sarcolemma. Characterization, calmodulin dependence, and partial purification. *J. Biol. Chem.* 256:3263–3270.

22. Weiss, B. 1983. Techniques for measuring the interaction of drugs with calmodulin. *Methods Enzymol.* 102:171–184.

23. Hidaka, H., and T. Tanaka. 1983. Naphthalenesulfonamides as calmodulin antagonists. *Methods Enzymol.* 102:185–194.

24. Dixon, M. 1953. The determination of enzyme inhibitor constants. *Biochem. J.* 55:170–171.

25. Niggli, V., E. S. Adunyah, and E. Carafoli. 1981. Acidic phospholipids, unsaturated fatty acids, and limited proteolysis mimic the effect of calmodulin on the purified erythrocyte Ca^{2+}-ATPase. *J. Biol. Chem.* 256:8588–8592.

26. Inagaki, I., H. Itoh, and H. Hidaka. 1983. Two types of calmodulin antagonists: a structurally related interaction. *Jpn. J. Pharmacol.* 33:183P. (Abstr.)

27. Harder, D. R., and N. Sperelakis. 1981. Bepridil blockade of Ca^{2+}-dependent action potentials in vascular smooth muscle of dog coronary artery. *J. Cardiovasc. Pharmacol.* 3:906–914.

28. Marshall, R. J., and A. W. Muir. 1981. The beneficial actions of bepridil in acute myocardial infarction in anesthetized dogs. *Br. J. Pharmacol.* 73:471–479.

29. Beaughard, M., M. Ferrier, C. Labrid, J. C. Lamar, J. Leboeuf, and P. Piris. 1982. Studies on the bradycardia induced by bepridil. *Br. J. Pharmacol.* 75:293–300.

30. Simaan, J. A., and D. M. Aviado. 1976. A comparative study between the cardiovascular effects of cetiedil, a new vasodilator, and papaverine and aminophylline. *J. Pharmacol. Exp. Ther.* 198:176–186.

31. Kawada, M., K. Satoh, and N. Taira. 1983. Profile of coronary vasodilator and cardiac actions of bepridil revealed by use of isolated, blood-perfused heart preparations of the dog. *J. Cardiovasc. Pharmacol.* 5:604–612.

32. Winslow, E., and K. A. Kane. 1981. Supraventricular antidysrhythmia and electrophysiological effects of bepridil, a new antianginal agent. *J. Cardiovasc. Pharmacol.* 3:655–667.

33. Narahara, K. A., W. Shapiro, J. Park, and I. Weliky. 1983. Anti-anginal effects of bepridil—a new calcium channel blocker—in patients with stable angina pectoris. *Clin. Res.* 31:15. (Abstr.)

34. Katz, R. J., R. Dibianco, E. Chesler, J. Alpert, J. Spann, and R. Costello. 1983. Sustained beneficial effect of bepridil in exertional angina. *Circulation.* 68(Supp. III):1607. (Abstr.)

35. Flammang, D., M. Wayneberger, R. Paillet, and P. Coumel. 1983. Electrophysiological profile of bepridil, a new anti-anginal drug with Ca^{2+}-blocking properties. *Eur. Heart J.* 4:647–654.

36. Davy, J. M., J. F. Laine, C. Sebag, and G. Motte. 1983. Bepridil, a new calcium antagonist for the treatment of ventricular tachycardia. *Circulation.* 68(Supp. III):1241. (Abstr.)

37. Rowland, E., W. McKenna, and D. Krickler. 1983. Electrophysiological and antiarrhythmic effects of bepridil in reentry AV tachycardia: comparison with verapamil and ajmaline. *Circulation.* 68(Supp. III):1242. (Abstr.)

38. Daly, K., J. W. Upward, S. Campbell, L. Atkinson, and D. E. Jewitt. 1983. The acute haemodynamic, metabolic, and electrophysiologic effects of intravenous bepridil in man. *Circulation.* 68(Supp. III):1006. (Abstr.)

39. Levy, S., M. Metge, R. Cointe, G. Fangere, B. Valeix, and R. Gerard. 1983. Treatment of recurrent ventricular tachycardias with bepridil, a slow and fast channel blocking agent. *Circulation.* 68(Supp. III):1087. (Abstr.)

40. Ravdin, J. I., N. Sperelakis, and R. L. Guerrant. 1982. Effect of ion channel inhibitors on the cytopathogenicity of *Entamoeba hystolytica. J. Infect. Dis.* 146:335–340.

41. Berkowitz, L. R., and E. P. Orringer. 1981. Effect of cetiedil, an in vitro anti-sickling agent, on erythrocyte membrane cation permeability. *J. Clin. Invest.* 68:1215–1220.

42. Schmidt, W. F., T. Asakura, and E. Schwartz. 1982. Effect of cetiedil on cation and water movement in erythrocytes. *J. Clin. Invest.* 69:589–594.

43. Asakura, T., S. T. Ohnishi, K. Adachi, M. Ozguc, K. Hashimoto, M. Singer, M. O. Russell, and E. Schwartz. 1980. Effect of cetiedil on erythrocyte sickling: new type of antisickling agent that may affect erythrocyte membranes. *Proc. Natl. Acad. Sci. USA.* 77:2955–2959.

44. Benjamin, L. J., G. Kokkini, and C. M. Peterson. 1980. Cetiedil: its potential usefulness in sickle cell disease. *Blood.* 55:265–270.

45. Brewer, G. J., U. Bereza, I. Mizukami, J. C. Aster, and L. F. Brewer. 1981. Drug action hypothesis: how membrane expansion, calmodulin inhibition, and sickle cell therapy relate. *In* The Red Cell, Fifth Ann Arbor Conference. G. J. Brewer, editor. Alan R. Liss, Inc., New York. 187–207.

46. Levine, S. N., L. R. Berkowitz, and E. P. Orringer. 1984. Cetiedil inhibits calmodulin stimulated enzyme activity. *Biochem. Pharmacol.* 33:581–584.

47. Wolach, J. B., T. D. Coates, D. Y. Tzeng, R. L. Baehner, and L. A. Boxer. 1983. Modulation of polymorphonuclear leukocyte functions by cetiedil. *Blood.* 62:274–279.

48. Wise, B. C., D. B. Glass, C.-H. J. Chou, R. L. Raynor, N. Katoh, R. C. Schatzman, R. S. Turner, R. F. Kibler, and J. F. Kuo. 1982. Phospholipid-sensitive Ca^{2+}-dependent protein kinase from heart. II. Substrate specificity and inhibition by various agents. *J. Biol. Chem.* 257:8489–8495.

49. Tanaka, T., T. Ohmura, T. Yamakado, and H. Hidaka. 1982. Hydrophobic interaction of the Ca^{2+}-calmodulin complex with cal-

modulin antagonists: naphthalenesulfonamide derivatives. *Mol. Pharmacol.* 22:403–407.

50. Mazzei, G., R. C. Schatzman, R. S. Turner, W. R. Vogler, and J. F. Kuo. 1984. Phospholipid sensitive Ca^{2+}-dependent protein kinase inhibition by R-24571, a calmodulin antagonist. *Biochem. Pharmacol.* 33:125–130.

51. Stewart, A. A., T. S. Ingebretsen, and P. Cohen. 1983. The protein phosphatases involved in cellular regulation. 5. Purification and properties of a Ca^{2+}/calmodulin dependent phosphatase (2B) from rabbit skeletal muscle. *Eur. J. Biochem.* 132:289–295.

52. Moore, P. B., and J. R. Dedman. 1982. Calcium-dependent protein binding to phenothiazine columns. *J. Biol. Chem.* 257:9663–9667.

53. Epstein, P. M., K. Fiss, R. Hachisu, and D. M. Andrenyak. 1982. Interaction of calcium antagonists with cyclic AMP phosphodiesterases and calmodulin. *Biochem. Biophys. Res. Commun.* 105:1142–1149.

54. Andersson, A., T. Drakenberg, E. Thalin, and S. Forsen. 1983. A ^{113}Cd and ^{1}H NMR study of the interactions of calmodulin with D600, trifluoperazine, and some other hydrophobic drugs. *Eur. J. Biochem.* 134:459–465.

55. Bostrom, S.-L., B. Ljung, S. Mardh, S. Forsen, and E. Thulin. 1981. Interaction of the antihypertensive drug felodipine with calmodulin. *Nature (Lond.).* 292:777–778.

56. Chafouleas, J. G., W. E. Bolton, H. Hidaka, A. E. Boyd, and A. R. Means. 1982. Calmodulin and the cell cycle: involvement in regulation of cell-cycle progression. *Cell.* 28:41–50.

57. Raess, B. U., and F. F. Vincenzi. 1980. Calmodulin activation of red blood cell (Ca^{2+} + Mg^{2+})-ATPase and its antagonism by phenothiazines. *Mol. Pharmacol.* 18:253–258.

58. Murakami, T., M. Hatanaka, and T. Murachi. 1981. The cytosol of human erythrocyte contains a highly Ca^{2+}-sensitive thiol protease (calpain I) and its specific inhibitor protein (calpastatin). *J. Biochem.* 90:1809–1816.

59. Siefring, G. E., A. B. Apostol, P. T. Velasco, and L. Lorand. 1978. Enzymatic basis for the Ca^{2+}-induced cross-linking of membrane proteins in intact human erythrocytes. *Biochemistry.* 17:2598–2604.