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A CALMODULIN-LIKE Ca^{2+} RECEPTOR IN THE Ca^{2+} CHANNEL

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The voltage-dependent Ca^{2+} channel, or slow channel, transports Ca^{2+} across the cell membrane and is regulated by intracellular [Ca^{2+}] (1). It must, therefore, bind Ca^{2+} itself, and some domain or region of the Ca^{2+}-channel protein must be a Ca^{2+}-binding protein. Calmodulin (CDR) is perhaps the most universal Ca^{2+}-dependent regulatory protein and we have studied it as a model for the Ca^{2+} receptor in the Ca^{2+} channel. We have reported that some Ca^{2+}-antagonist drugs (Ca^{2+} channel blockers), said to be specific for the Ca^{2+} channel, bind to calmodulin (2). We have also shown that calmodulin antagonists (CDR-ANT) can act like Ca^{2+}-antagonists (Ca-ANT) and block the Ca^{2+} channel (3). Recently, allosteric interactions among Ca-ANT drug-binding sites on the Ca^{2+} channel have been reported (4, 5). The present study demonstrates allosteric interactions among the Ca-ANT–binding sites of calmodulin. This provides further, indirect, evidence of a Ca^{2+}-binding protein, similar to but probably distinct from calmodulin, serving as a regulator of the Ca^{2+} channel and a receptor for Ca-ANT drugs. With this information we propose a model of the Ca^{2+} channel and the mechanism of action of Ca-ANT.

RESULTS

Ca^{2+} binding to calmodulin is known to produce large structural changes including the exposure of 3–4 hydrophobic ligand-binding sites (7). The dihydropyridine Ca-ANT, felodipine, binds one to two of these sites on CDR with a *Kd* of 1–10 μM (8). The fluorescence spectra of felodipine (Fig. 1, inset) is not changed by the addition of CDR. The addition of Ca^{2+} (but not Mg^{2+}), only in the presence of CDR, produces a twofold fluorescence increase (Fig. 1, inset, curve 2). This suggests that Ca^{2+} binding to Ca^{2+}-specific sites on CDR exposes felodipine binding sites and that felodipine binding can be monitored by this fluorescence increase. Addition of the CDR-ANT, R24571, or the Ca-ANT, diltiazem, to Ca^{2+}-CDR-felodipine can produce a further fluorescence increase (Fig. 1, inset curve 3) only in the presence of both Ca^{2+} and CDR. This suggests that these drugs do not interact with free felodipine but bind to a Ca^{2+}-CDR complex to increase felodipine binding to CDR.

In the same concentration range where R24571 and diltiazem bind to CDR (2), we find that they produce an increase in CDR-felodipine fluorescence; at higher concentrations this fluorescence increase is reversed (6). Initially, these drugs bind to sites on CDR other than the felodipine-binding sites. Through an allosteric mechanism they increase the number and/or affinity of felodipine-binding sites, resulting in the observed fluorescence increase. At higher concentrations presumably they can bind to the felodipine-binding sites and competitively displace felodipine, resulting in the observed fluorescence decrease.

A titration of felodipine with CDR is shown in Fig. 1.

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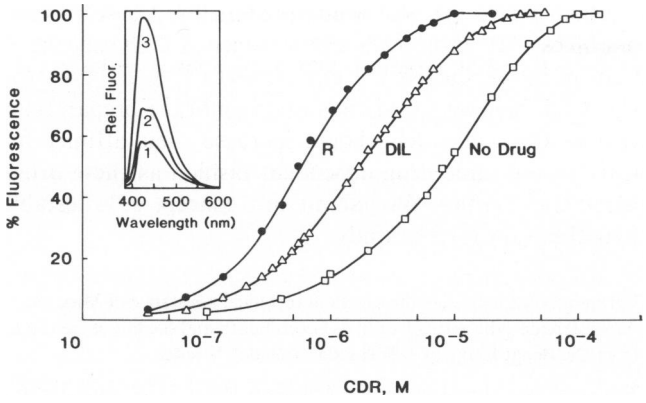

FIGURE 1 Effect of Ca^{2+} antagonists and calmodulin antagonists on felodipine binding to calmodulin. The percent of the total fluorescence increase in felodipine as a function of added calmodulin is shown in the presence of 2 μM R24571 (●—●), 500 μM diltiazem (△—△) and in the absence of added drug (□—□). The total fluorescence increases were 3.1-, 10.8-, and 2.2-fold, respectively. Each cuvette contained 5×10^{-7} M felodipine in 10 mM MOPS, 90 mM KCl, 2 mM EGTA buffer with 2.9 mM $CaCl_2$ added, and was titrated with CDR in the presence or absence of drug. *Inset*. Fluorescence spectra of 1 μM felodipine (curve 1) in above buffer followed by successive additions of 2 μM calmodulin (curve 1), 3 mM $CaCl_2$ (curve 2), and 2 μM R24571 (curve 3).

Half-maximal binding occurs at 8.5 μM (as estimated from this fluorescence increase), in good agreement with the estimated 1–10 μM *K*d determined by NMR (8). Adding 2 μM R24571 or 500 μM diltiazem shifts the titrations to the left of the control (no drug) curve (Fig. 1). This indicates that R24571 and diltiazem can bind to CDR and increase the apparent affinity of CDR for felodipine by 15-fold with R24571 and fivefold with diltiazem. The inactive isomer of diltiazem, L-*cis*-diltiazem, and verapamil are much less effective than diltiazem in producing these effects. These studies clearly indicate that the CDR-ANT, R24571, and the Ca-ANT, diltiazem, can act through an allosteric mechanism to increase the apparent affinity of calmodulin for felodipine.

These allosteric interactions among drug-binding sites on CDR may affect its mechanisms of action, because these hydrophobic drug-binding sites also appear to be the sites at which calmodulin binds the proteins it activates. The degree of occupancy of these hydrophobic ligand-binding sites on CDR by drugs, proteins, or by endogenous regulators may determine the action and specificity of CDR in regulating various Ca^{2+}-dependent processes within the cell.

Studies of 1,4 dihydropyridine binding to brain and heart sarcolemmal membranes suggest that allosteric interactions occur among Ca-ANT drug-binding sites on Ca^{2+} channel proteins (4, 5). In fact, diltiazem (but neither verapamil nor L-*cis*-diltiazem) potentiates dihydropyridine-binding to these membranes (5). We have shown further that the CDR-ANT, R24571, can potentiate the action of some Ca-ANT in altering Ca^{2+} currents (3). These studies suggest that similar allosteric mechanisms may exist among drug-binding sites on both the Ca^{2+} channel and on CDR.

At present it is difficult to say that CDR, itself, is the Ca^{2+} receptor of the Ca^{2+} channel, because the affinity of Ca-ANT for isolated, purified CDR is 10–1,000 times less than their affinity for the Ca^{2+} channel protein. Instead, we propose that a Ca^{2+}-binding protein, similar to CDR but having a higher affinity for Ca-ANT drugs, is a Ca^{2+} receptor of the Ca^{2+} channel and is a pharmacological receptor for some Ca^{2+}-antagonist drugs. In support of this hypothesis we find that both our model Ca^{2+}-binding protein, CDR, and the Ca^{2+}-channel protein(s) bind Ca^{2+} in the submicromolar range and act to facilitate their regulatory functions; both bind and are inhibited by some Ca-ANT and CDR-ANT drugs; and both exhibit allosteric interactions among their drug-binding sites.

The Ca^{2+} channel is thought to be a multisubunit protein (~200,000 M_r) that traverses the cell membrane. It presumably has Ca^{2+}-binding sites on both its extracellular side (Fig. 2, site 1) and its intracellular side (site 2). Ca-ANT exert their effects at site 2 (9). The channel is inactivated by a rise in $[Ca^{2+}]_i$ and therefore by a Ca^{2+}-binding protein on its intracellular side. Our working hypothesis suggests that site 2 is a calmodulin-like Ca^{2+} binding protein (CBP) which is the receptor for Ca-ANT drugs and the Ca^{2+} dependent gate of the channel. Under

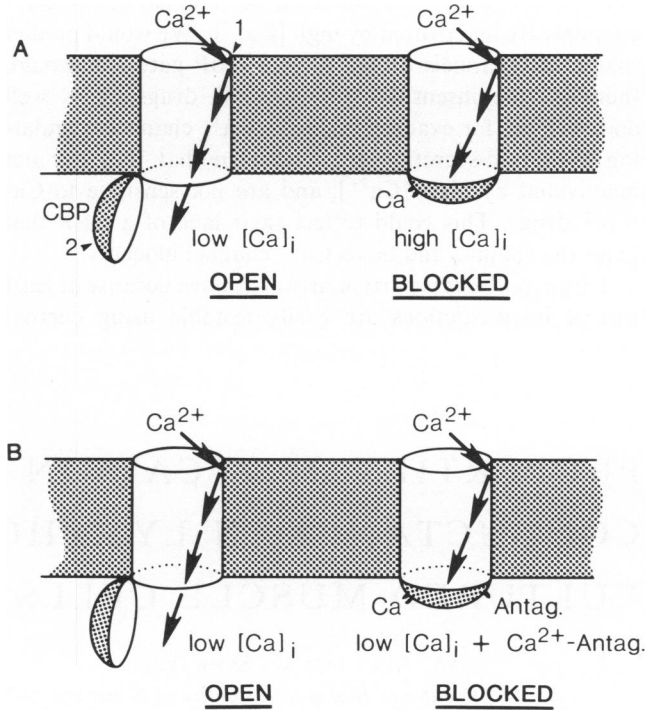

FIGURE 2 A schematic representation of the possible role of a calmodulin-like Ca^{2+} binding protein (CBP) as a pharmacological receptor for Ca^{2+} antagonist drugs and as the Ca^{2+}-dependent gate of the Ca^{2+} channel. The membrane is assumed to be depolarized.

conditions of low $[Ca^{2+}]_i$, pCa > 7.0, the CBP gate (site 2) is open and the channel can be opened in a voltage-dependent manner. As $[Ca^{2+}]_i$ rises (pCa < 7.0), Ca^{2+} binds to this CBP (site 2), closing or inactivating the channel (Fig. 2 *A*).

Using CDR as a model for the CBP gate of the channel, we recall that protein or drug binding to CDR is able to increase its affinity for Ca^{2+} by nearly tenfold (10). Our unpublished results show that R24571 binding to CDR can increase the Ca^{2+} affinity of the Ca^{2+} sites that regulate felodipine binding to CDR by as much as 50-fold.

If the CBP of site 2 is similar to CDR in this regard also, then its binding of Ca-ANT could increase its affinity for Ca^{2+} enough to bind Ca^{2+}, close the channel, and keep it closed even under low $[Ca^{2+}]_i$ conditions (Fig. 3 *B*). Thus, Ca-ANT may bind to the CBP gate of the channel, potentiate Ca^{2+} binding to this protein, and thereby facilitate the action of $[Ca^{2+}]_i$ in closing or inactivating the channel. Under these circumstances, Ca^{2+} channel blockers are perhaps more Ca^{2+}- agonists than Ca^{2+}-antagonists, since they facilitate the action of intracellular Ca^{2+} with respect to channel blockade. Ca-ANT could vary in the affinity and/or Ca^{2+} dependence of their binding to this CBP gate and in their ability to increase its affinity for Ca^{2+}, which might explain the differences in effectiveness and frequency-dependence of various Ca-ANT's. Different Ca-ANT may have different allosterically-related binding sites on this CBP, explaining the ability of some drugs to potentiate the binding and action of other drugs.

Consistent with our hypothesis, we note that not all Ca^{2+} channels are inactivated by high $[Ca^{2+}]_i$. We would predict that these channels do not have a CBP gate and would, therefore, be insensitive to Ca-ANT drugs. It is well documented, for example, that the Ca^{2+} channels regulating excitation-secretion are unique in that they are not inactivated by high $[Ca^{2+}]_i$ and are not sensitive to Ca-ANT drugs. This could reflect their lack of a CBP that gates the channel and binds Ca^{2+} channel blockers.

This hypothesis is particularly attractive because at least two of its predictions are easily testable using current methodologies. (*a*). We would predict that Ca-ANT cannot block Ca^{2+} currents in the presence of high concentrations of intracellular Ca^{2+} chelators (EGTA, EDTA). (*b*) Ca-ANT binding to sarcolemma membrane preparations rich in Ca^{2+} channels should increase their affinity for Ca^{2+} in the same drug-dependent fashion as these drugs block Ca^{2+} influx. We submit this concept as a testable hypothesis for further study.

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PROPERTIES OF A CATION CHANNEL OF LARGE UNIT CONDUCTANCE IN LYMPHOCYTES, MACROPHAGES AND CULTURED MUSCLE CELLS

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Using the patch-clamp method (1, 2), we have identified a cation channel with novel properties in normal and transformed nonexcitable cells as well as in excitable cells. For experiments with nonexcitable cells, cultured FO-cells, thymocytes, and peritonal macrophages of mouse were used.

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