Metal Ions as Allosteric Regulators of Calmodulin*

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Previously we have shown that the fluorescence of the dihydropyridine calcium antagonist felodipine provides an accurate means of monitoring the formation of an allosterically potentiated conformer of calmodulin (Mills, J. S., and Johnson, J. D., (1985) Biochemistry 24, 4897–4903). Characteristic of this conformer is the abolition of cooperativity among the two felodipine-binding sites on calmodulin and a 20-fold increase in the apparent affinity of calmodulin for felodipine. In the present study, we find that the metal cations La³⁺, Tb³⁺, Pb²⁺, and Cd²⁺ are all capable of abolishing the cooperativity (Hill coefficient = 2.0) among the two felodipine-binding sites on calmodulin and can increase the apparent affinity of calmodulin for felodipine by approximately 20-fold. These effects are seen either in the presence or absence of calcium and are half-maximal at 8, 12, 22, and 1000 μM, respectively. Zinc and H⁺ are capable of producing similar potentiations of felodipine binding (half-maximal at 570 μM, and pH 5.8), but only in the presence of calcium. In each case, the calcium-binding sites of calmodulin must be occupied (by calcium, La³⁺, Tb³⁺, Pb²⁺, or by Cd²⁺) before these metals can bind to sites which are distinct from the calcium-binding sites to produce the active conformer of calmodulin which exhibits enhanced affinity for felodipine.

Mercury and copper can compete with these potentiating metal cations on calmodulin and produce an inactivation of this active calmodulin conformer. These studies suggest that some metals including La³⁺, Tb³⁺, Pb²⁺, Cd²⁺, Zn²⁺ and protons are capable of binding to a calcium-calmodulin complex and forming an allosterically active species of calmodulin which cannot be maintained by physiological concentrations of calcium ions alone. Mercury and copper, on the other hand, are capable of inactivating this active calmodulin conformer independent of the presence of calcium on calmodulin.

These findings are examined in terms of the mechanism of action of calmodulin and its possible role in heavy metal toxicity.

Calmodulin is a ubiquitous calcium-binding protein that has now been implicated in conferring calcium sensitivity to over 30 different target proteins (see Refs. 1–4, for review). With calcium binding to the four calcium-binding sites on calmodulin, large structural changes including increases in α-helix and intrinsic tyrosine fluorescence occur. Perhaps the most significant calcium-dependent event to occur on the surface of calmodulin is the exposure or formation of hydrophobic drug/protein-binding sites (5–7). These interfacial sites are thought to be the sites where calmodulin interacts with its target proteins and with inhibitory drugs (calmodulin antagonists). Felodipine (4-(2,3-dichlorophenyl)-1,4-dihydropyridine-2,6-dimethyl-3,5-dicarboxylic 3-ethyl ester and 5-methyl ester) is perhaps the most potent member of the powerful dihydropyridine calcium channel blocking drugs (27, 28). Unlike most other therapeutically useful dihydropyridines, felodipine is fluorescent. It binds to calcium channels on purified sarcolemma fractions with affinities in the sub-nanomolar range and has recently been shown to bind to calmodulin in a calcium-dependent manner (8, 9, 29). It has been suggested that felodipine’s interaction with calmodulin may contribute to its potency as a vasodilator (29). Recently, we have shown that some of these calcium-dependent drug-binding sites are allosterically related (8, 9) and that drug binding to some site(s) can potentiate felodipine binding to a distinct site by producing a 20-fold increase in its apparent affinity. We have shown that the fluorescence increase which occurs with the binding of felodipine provides an accurate means of monitoring these allosteric interactions among calmodulin’s drug-binding sites (9).

In addition to drug-binding sites, calmodulin has metal-binding sites. Not only calcium but La³⁺, Tb³⁺, Pb²⁺, Sm³⁺, Sr²⁺, Hg²⁺, Cd²⁺, Zn²⁺, and Mn²⁺ have been shown to interact with calmodulin (10–15). Some of these metals can compete for the calcium-binding sites on calmodulin while others may have effects on calmodulin’s conformation beyond those produced by calcium alone (14). Recently, calmodulin has been suggested to be a mediator of some of the toxic effects of these metals in heavy metal toxicity (13). Several authors have shown that some of these metals can activate calmodulin-dependent phosphodiesterase in the absence of calcium (13, 16) while others may inhibit in the presence of calcium (17, 18). Moreover, a close correlation between metal toxicity and metal binding to calmodulin has been demonstrated (18). In addition, many of these metals affect lymphocyte mitogenesis (19), a process known to be calcium-dependent (20).

In this paper we examine the effects of metal cations, including La³⁺, Tb³⁺, Cd²⁺, Pb²⁺, Zn²⁺ and H⁺, in producing the conformation of calmodulin which is allosterically potentiated for binding felodipine. Furthermore, we examine the effects of Hg²⁺ and Cu²⁺ in displacing these other metals and inactivating this active conformer of calmodulin.

Our findings suggest that if interactions of some of these metals and calmodulin occur in vivo, they could dramatically affect calmodulin’s regulation of calcium-dependent events.
within the cell and produce significant biological consequences.

**EXPERIMENTAL PROCEDURES**

**Equilibrium Dialysis**—Measurements were conducted in a buffer of 10 mM Mops, pH 7.0, 90 mM KCl with 2 mM EGTA and 3 mM Ca$^{2+}$ or 1 mM La$^{3+}$ and 1 mM Ca$^{2+}$. [3H]Felodipine of constant specific activity was added to the solution (50 ml) outside the dialysis bag which contained 3 ml of 1 μM calmodulin. Specific activity was determined using a milimolar extinction coefficient at 370 nm of 6.4 in 95% ethanol.

**Fluorescence Studies**—Buffers were sufficiently free of Ca$^{2+}$ so as not to produce any fluorescence increase in the presence of both calmodulin and felodipine. Since we have previously shown that half-maximal felodipine binding occurs at 1 μM Ca$^{2+}$ (9), free Ca$^{2+}$ was less than 1 μM. This was verified by atomic absorption spectroscopy. Fluorescence titrations were carried out in 10 mM Mops, pH 7.0, unless otherwise indicated using excitation at 365 nm and emission at 445 nm.

Hill plots were analyzed by an iterative linear regression Hill plot program. $B_{	ext{max}}$ was determined by the best fit of the data to the Hill equation. Felodipine and [3H]felodipine were gifts of A. B. Hassle Pharmaceutical, Mölndal, Sweden. All other chemicals were reagent grade. Calmodulin was purified from bovine testes as previously described (10).

**RESULTS**

Previously, we have shown two felodipine-binding sites on calmodulin. These sites exhibit cooperativity in their drug binding. Calmodulin antagonists (including prenylamine and R24571) bind to calmodulin and allosterically potentiate felodipine binding to its remaining site by producing a large increase in its affinity (8, 9). Some metals are capable of producing similar enhancements of felodipine binding and felodipine-calmodulin fluorescence. The inset of Fig. 1 shows the fluorescence spectra of felodipine in the presence of calmodulin (spectra 1), after the addition of 1 mM calcium (spectra 2), and after the subsequent addition of 300 μM lanthanum chloride. Calcium produces a 40% increase in felodipine-calmodulin fluorescence, and the subsequent addition of lanthanum produces a large additional 4.0-fold increase in fluorescence. In the absence of added calcium, lanthanum alone produces a 6.2-fold increase in fluorescence with the concentration dependence shown in Fig. 1. Lead and cadmium produce similar enhancements, both in the presence and in the absence of added calcium, with the concentration dependence shown in Fig. 1. Zinc produces a 4.5-fold fluorescence increase over calcium alone and no fluorescence increase in the absence of calcium. Excitation and emission wavelengths were at 365 and 445 nm, respectively.

The abbreviations used are: Mops, 3-(N-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid.
fluorescence in the absence of calmodulin.

Previously, we have shown that the fluorescence increase produced by felodipine binding to calmodulin can be used to quantitate felodipine binding in the presence and absence of potentiating drugs (9). A similar direct correlation between $^3$H felodipine binding and the felodipine fluorescence increase produced by binding is shown in Fig. 2 (in the presence and absence of lanthanum). An 8-fold increase in fluorescence corresponds to 1 mol of felodipine binding per mol of calmodulin. Thus, the increase in felodipine fluorescence produced by these potentiating metals is due simply to an increase in calmodulin's affinity for felodipine and therefore more felodipine molecules binding to calmodulin, as verified by these $[^3H]$felodipine binding studies. This figure was used as a standard curve to quantitate felodipine binding in the following fluorescence experiments.

Hill plots of felodipine titrations of calmodulin alone and calmodulin potentiated by zinc + calcium or by lanthanum + calcium are shown in Fig. 3. Felodipine alone exhibits a Hill coefficient of 2.1 ($r = 0.999$), indicative of cooperativity among the two felodipine-binding sites. A $B_{\text{max}}$ of 2.0 mol/mol was determined by best fit analysis of this data and a $K_{d}$ of 8 $\mu$M was found. In the presence of zinc + calcium or lanthanum + calcium, the Hill coefficient was reduced to 0.96 ($r = 0.98$) or 0.83 ($r = 0.97$ with $K_{d}$ of 0.5 and 0.2 $\mu$M, respectively. A $B_{\text{max}}$ of 2.0 was determined with both zinc and lanthanum. These potentiating metals abolish the cooperativity among calmodulin's two felodipine-binding sites. Calmodulin titrations of felodipine with and without a metal were used to determine the effect of potentiating metals on calmodulin's affinity for felodipine. In the absence of metal, calmodulin binds felodipine with a $K_{d}$ of 8.0 $\mu$M, while in the presence of zinc + calcium or lanthanum, the $K_{d}$ is decreased approximately 20-fold to 0.4 $\mu$M (Fig. 4). These potentiating metals, therefore, produce a very dramatic increase in calmodulin's affinity for felodipine.

Zinc is the only metal which requires calcium before it will dramatically potentiate felodipine binding to calmodulin. This allowed us to determine the calcium dependence of zinc potentiation. Calcium titrations of felodipine-calmodulin at various concentrations of zinc are shown in Fig. 5. The calcium dependence is approximately the same at concentrations of zinc which produce one-quarter maximal, one-half maximal, and maximal stimulation of felodipine binding. The similarity of the calcium dependence of potentiation over a wide range of zinc concentrations suggests that zinc is not very competitive with calcium at the calcium-binding sites. Zinc titrations of felodipine-calmodulin at 1 mM and 50 $\mu$M calcium and at 5 mM strontium are shown in Fig. 6. At lower calcium concentrations (50 $\mu$M), zinc potentiates felodipine fluorescence at low concentrations but inhibits it at higher calcium. At higher calcium (1 mM), zinc only potentiates felodipine fluorescence over the range tested. This suggests that zinc, at a higher concentration, is able to compete with calcium (at lower concentrations), displacing it from calmodulin and thus preventing felodipine binding and fluorescence enhancement. At higher calcium concentrations (1 mM), zinc is less able to effectively displace calcium and produce this fluorescence decrease. Strontium is known to substitute for
Fig. 5. Effect of calcium on the zinc-mediated potentiation of calmodulin-felodipine fluorescence. The fluorescence increase over the fluorescence of felodipine alone (f/f_0), in the presence of 230 \(\mu\)M zinc (A–A), 575 \(\mu\)M zinc (O–O), and 3.55 \(\mu\)M zinc (□–□), is shown as a function of added calcium. The buffer was the same as in Fig. 1 with 1 \(\mu\)M calmodulin and 1 \(\mu\)M felodipine.

Fig. 6. The effect of zinc in potentiating calmodulin-felodipine fluorescence at different calcium and strontium concentrations. The total fluorescence change is shown as a function of added zinc for 1 mM calcium (A–A), 50 \(\mu\)M calcium (Δ–Δ), and 5 mM strontium (□–□). The buffer was that used in Fig. 1 with 1 \(\mu\)M felodipine and 1 \(\mu\)M calmodulin.

calcium on calmodulin (30). It has a lower affinity for calmodulin and produces a small increase in felodipine-calmodulin fluorescence (1.2-fold) similar to calcium. Zinc initially potentiates the strontium-supported increase in fluorescence and then displaces strontium to prevent felodipine binding at higher zinc concentrations (see Fig. 6). These data suggest that high concentrations of zinc can compete with the calcium (and strontium)-binding sites on calmodulin, under conditions of low calcium. Thus, while calcium cannot effectively compete with the zinc-binding site(s) which produce potentiation, high concentrations of zinc can compete with the calcium sites on calmodulin.

Copper and mercury produce no potentiation of felodipine-calmodulin fluorescence either in the absence or presence of calcium. Instead, they decrease the fluorescence increases produced by the potentiating metals. Fig. 7 shows the effects of copper and mercury in reversing the potentiated states produced by zinc + calcium, lanthanum, and lanthanum + calcium. Copper half-maximally inhibits each of these potentiated states near 50 \(\mu\)M. Mercury is less effective, half-maximally inhibiting each of these potentiated states near 2 mM. The concentration range over which both copper and mercury produce the reversal of the lanthanum-potentiated state is essentially the same in the presence of 1 mM calcium as in the absence of added calcium. Similar results were found with lead. Copper half-maximally inhibits the lead-potentiated state (300 \(\mu\)M lead) at 50 \(\mu\)M both in the presence and absence of 1 mM calcium. The calcium dependence of the zinc-potentiated state (3.6 mM zinc) is not affected (half-maximal ~50 \(\mu\)M calcium) by the presence of 50 \(\mu\)M copper, while this concentration of copper does reduce the total amount of felodipine bound in the presence of zinc ([f/f_0 = 2.3]). Furthermore, in the presence of submaximal potentiating metal (55 \(\mu\)M Pb2+ and 15 \(\mu\)M La3+), copper is more effective in inactivating the potentiated state (half-maximal at 14 \(\mu\)M copper) than in the presence of higher concentrations of potentiator (see Fig. 6). Similar results were found either in the presence or absence of 1 mM added calcium. Moreover, 50 \(\mu\)M copper and 2 mM mercury shift the concentration dependence of the lanthanum-induced increase to the right from 8 \(\mu\)M (as in Fig. 1) to 50 and 60 \(\mu\)M, respectively. These data are consistent with both copper and mercury binding competitively at the potentiating metal-binding site(s) and not at the calcium-binding sites of calmodulin.

Calcium itself at concentrations well above where it binds to the calcium-binding sites on calmodulin is able to produce a potentiation of felodipine binding. Calcium at concentrations above the millimolar range produces a 5-fold increase in calmodulin-felodipine fluorescence which is half-maximal at 50 mM calcium. This suggests that calcium is 5000-fold less effective than La3+ and 100-fold less effective than zinc in binding to those potentiating sites on calmodulin.

Protons are also able to produce this highly fluorescent potentiated state of felodipine-calmodulin. Fig. 7 shows pH titrations of felodipine and of felodipine-calmodulin in the presence and absence of calcium. Felodipine alone and felodipine-calmodulin in the absence of calcium were not affected...
by pH over the range tested. Titrations of felodipine-calmodulin in the presence of calcium, however, showed a sharp pH dependence, with maximal potentiation near pH 4.75. These data suggest that concentrations of protons in this range can act in a similar manner as some of the metal cations in potentiating felodipine binding to calmodulin. Felodipine titrations of calmodulin + calcium at pH 4.75 verified that this was the case. Hill plots of these data (not shown) indicated that the Hill coefficient was reduced from 2.0 at pH 7.0 to 0.96 ($r = 0.996$) at pH 4.75 with a $K_{Hill}$ of approximately 1.7 mol/mol and $K_{Hill} = 4 \mu M$. Calmodulin titrations of felodipine at pH 4.75 indicate that the apparent affinity is increased approximately 14-fold relative to pH 7.0 (data not shown).

Thus, protons are capable ofabolishing the cooperativity among felodipine-binding sites and increasing the affinity of calmodulin for felodipine in a manner analogous to metals and potentiating drugs. Calcium is more effective in producing an increase in calmodulin-felodipine fluorescence as the proton concentration is reduced. At pH 5.5, calcium produces a 2.7-fold increase (over felodipine alone) which is half-maximal at 3.5 $\mu M$, while at pH 4.0, calcium produces a 2.9-fold increase which is half-maximal at 425 $\mu M$.

**DISCUSSION**

Previously, we have shown that allosteric interactions occur among calmodulin’s drug-binding sites (8, 9). Drugs, including the calmodulin inhibitors R24571 (calmidazolium), and pentyamine, can bind to one of the two felodipine-binding sites on calmodulin, abolish the cooperativity among these sites, and dramatically increase the affinity of felodipine at its remaining binding site (9). The fluorescence of the dihydro-pyridine calcium channel blocker, felodipine, provides an accurate means of monitoring the binding of ligands which potentiate felodipine binding by allosterically increasing the number of calmodulin molecules in the active conformation.

In the present study, we describe the effects of various metals in potentiating felodipine binding to calmodulin. La$^{3+}$, Tb$^{3+}$, Pb$^{2+}$, and Cd$^{2+}$ act in a manner analogous to calmodulin antagonists and dramatically potentiate felodipine binding to calmodulin. These metals cause potentiation over a similar concentration range in the absence of added calcium and in the presence of 1 mM calcium, suggesting that they stabilize this active conformer of calmodulin by binding to sites other than the calcium-binding sites. Zinc also produces a strong potentiation but only in the presence of calcium. With each of these metals and with zinc + calcium, the fluorescence increase results from a large increase in the affinity of felodipine for calmodulin which occurs concomitant with the abolition of cooperativity among the two felodipine-binding sites (Hill coefficient was reduced from 2.0 to 1.0). Titrations of felodipine with calmodulin indicate that the affinity of calmodulin for felodipine is increased approximately 20-fold in the presence of potentiating metals.

In the case of calmodulin antagonist drug-induced potentiation of felodipine binding, the potentiating drugs bind to calmodulin only in the presence of calcium and produce their potentiated state by binding to sites which are distinct from the calcium-binding sites on calmodulin (8, 9). The active conformer produced by these metals is kinetically identical to that produced by drugs + calcium. It is, therefore, likely that these metals require occupancy of the calcium-binding sites before they can bind to distinct site(s) and produce this active conformation of calmodulin. With zinc, the requirement for calcium on the calcium-binding sites before zinc can bind to the potentiation site is obvious. La$^{3+}$, Tb$^{3+}$, Pb$^{2+}$, and Cd$^{2+}$ can bind both to the calcium-binding sites and the potentiation site(s) to produce potentiation in the absence of calcium. Consistent with this, Chao et al. (13) have shown that La$^{3+}$, Tb$^{3+}$, Pb$^{2+}$, and Cd$^{2+}$ are all effective in displacing calcium from calmodulin, promoting an increase in its intrinsic tyrosine fluorescence and modulating its activation of phosphodiesterase. Zinc and manganese, on the other hand, are not very effective in displacing calcium and producing the structural changes produced by calcium binding (13). Zinc and manganese, therefore, require calcium before they can produce the active calmodulin conformer. Occupancy of the calcium-binding sites by La$^{3+}$, Tb$^{3+}$, Pb$^{2+}$, Cd$^{2+}$, or by calcium appears to be necessary before any of these metals can bind to the sites where they produce potentiation. These metals, therefore, appear to be similar to the potentiating drugs in that they also require occupancy of the calcium-binding sites before they will bind and produce the active calmodulin conformer (9).

Our findings suggest that potentiating metals including La$^{3+}$, Tb$^{3+}$, Pb$^{2+}$, and Cd$^{2+}$ bind to sites that are distinct from the calcium-binding sites to produce an allosterically potentiated state of calmodulin. Copper and mercury compete with these metals at this site(s) and produce an inactivation of this active calmodulin conformer. Our evidence for these conclusions is as follows. 1) The concentration dependence of potentiation by La$^{3+}$, Tb$^{3+}$, Pb$^{2+}$, and Cd$^{2+}$ is not shifted by the presence or absence of 1 mM calcium. 2) Potentiation by zinc requires calcium, suggesting that distinct sites are involved for calcium binding and for potentiation. 3) The calcium dependence of potentiation by zinc is similar over a wide range of zinc concentrations, suggesting that over the range where zinc potentiates it does not compete for the calcium-binding sites. 4) Chao et al. (13) have shown that Pb$^{2+}$ and Cd$^{2+}$ are equally effective in displacing calcium from calmodulin, yet Pb$^{2+}$ is more than 50-fold more effective than Cd$^{2+}$ in potentiating felodipine binding. 5) Copper and mercury inactivate the La$^{3+}$-, Pb$^{2+}$-, and Zn$^{2+}$-potentiated state over the same concentration range irrespective of calcium concentration, suggesting that these metals compete for the metal ion potentiating site and not the calcium-binding sites. 6) The dependence of La$^{3+}$ potentiation is shifted to the right by the presence of Cu$^{2+}$ and Hg$^{2+}$, suggesting that Cd$^{2+}$ and Hg$^{2+}$ compete with the La$^{3+}$-potentiating site and not the calcium-
binding sites. 7) The calcium dependence of zinc potentiation is not shifted by the presence of half-maximally inhibiting copper (although the extent of potentiation is reduced), suggesting that copper inactivates by competing with the potentiating metal-binding site (zinc) and not at the calcium-binding site. 8) As the concentrations of potentiating metal (La\(^{3+}\), Pb\(^{2+}\)) are reduced, Cu\(^{2+}\) and Hg\(^{2+}\) are more effective in inactivating the potentiated state, independent of the presence of 1 mM added calcium. Again, this suggests that Cu\(^{2+}\) and Hg\(^{2+}\) compete at the metal ion potentiating site and not at the calcium-binding sites. 9) At very high concentrations, calcium itself can produce potentiation of felodipine binding which is similar to that seen with much lower concentrations of these metal cations, suggesting that at supraphysiological concentrations, calcium will occupy these metal ion potentiating sites. 10) Studies of terbium binding to calmodulin indicate that over the same concentration range where lanthanum, lead, cadmium, and zinc potentiate felodipine-calmodulin fluorescence (and over the range where copper decreases fluorescence), these metals will displace terbium. 2 This is consistent with these potentiating metals binding at the same ion binding sites on calmodulin. The effects of protons on this active conformer of calmodulin are similar to the action of zinc. In the presence of calcium, protons produce an activation followed by an inactivation of the active conformer at lower pH. The curve shown in Fig. 7 can be fit reasonably well assuming a maximum \(f_{\text{max}}\) of 8.0 (when all the molecules are in the active conformation), a \(K_{a}\) for activation of 5.15, and a \(K_{d}\) for inactivation of 4.65. Using these parameters, the maximum number of molecules in the active state occurs at pH 4.9 where approximately 40% of the calmodulin molecules are in the active conformation. Activation presumably occurs with protonation of carboxyl groups on the protein with \(K_{d}\) 5.15. Inactivation presumably occurs by displacement of calcium by protons at lower pH. Consistent with this, Andersson et al. (14) and Haiech et al. (23) have shown that protons can compete with calcium on calmodulin (\(K_{d}\) 4.4) in this range. Furthermore, calcium is 120-fold more effective in producing an increase in felodipine-calmodulin fluorescence at pH 5.3 than at pH 4.0. These findings are consistent with proton competition with calcium on calmodulin at low pH and can explain the inactivation of the active conformer, by displacement of calcium, below pH 4.9.

Our studies indicate that some metals are capable of not only occupying the calcium-binding sites of calmodulin, but also of supporting an allosterically potentiated conformer of calmodulin. This potentiation is produced by these metals binding to sites on calmodulin which are exposed or formed after calcium has bound. This active conformer is similar to the conformer produced by calcium and low concentrations of calmodulin antagonists (including R24571 and prenylamino acids). Previously, we have suggested that this conformer of calmodulin may represent a state of calmodulin which is activated for selective activation of some of calmodulin’s more than 30 target proteins (8, 9). Activation of calmodulin in the cell is the consequence of increased intracellular calcium levels and perhaps the presence of putative endogenous calmodulin substrates (9). Together these may produce active calmodulin conformers for the selective activation of calmodulin’s various target pro-

\(^{2}\) J. S. Mills and J. D. Johnson, unpublished observations.

Metal Ions and Calmodulin