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PERSPECTIVE

Ca^{2+} Regulation of Inositol 1,4,5-trisphosphate Receptors: Can Ca^{2+} Function without Calmodulin?

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ABSTRACT

All Ca^{2+} channels are regulated by Ca^{2+}, a feature that allows them to respond to their own activity and to the activities of neighboring Ca^{2+} channels. Inhibition by Ca^{2+} protects cells from potentially hazardous increases in cytosolic [Ca^{2+}], and stimulation can mediate facilitation and regenerative propagation of Ca^{2+} signals. Calmodulin is emerging as a key player in regulation of Ca^{2+} channels by Ca^{2+}, but its role is more complex and more beautiful than might have been imagined.

CaM, a small (148 residues), ubiquitous, and highly conserved Ca^{2+}-binding protein, is the sensor that links increases in cytosolic [Ca^{2+}] to changes in the activity of many enzymes and ion channels (Chin and Means, 2000). It is the "bottleneck" through which many Ca^{2+} signals pass before they can bring about changes in cellular activity. CaM forms a flexible dumbbell, and each of its two lobes includes a pair of Ca^{2+}-binding sites, each known as an "EF-hand" because this helix-loop-helix motif of about 30 residues (Fig. 1A) was first identified in a related protein, parvalbumin, where it is formed by the E- and F-helices (Ikura, 1996). More than 250 proteins use such EF-hands to reversibly bind Ca^{2+} (Celio et al., 1996). Within the loop of each EF hand, Ca^{2+} is cradled by seven oxygen atoms provided by backbone carbonyls, water, and the side chains of two conserved acidic residues, one at either end of the loop (Geiser et al., 1991) (Fig. 1B). Mutation of the first of these (Fig. 1B, D to A, red) produces an EF-hand with very low affinity for Ca^{2+}, probably at least 100-fold lower than the native site (Geiser et al., 1991; Xia et al., 1998). Such mutants of CaM have proven useful in resolving the roles of the two lobes of CaM in modulating Ca^{2+} channels. These CaM mutants are usually described with the defective Ca^{2+}-binding site(s) numbered, hence CaM_{12} is mutated in the first two (N-terminal) EF hands.

The N- and C-terminal lobes of CaM are similar but not identical. In the absence of Ca^{2+}, the C-terminal sites are more open than those in the N-lobe, they have ~10-fold greater affinity for Ca^{2+}, and Mg^{2+} dissociates more rapidly from them. The latter is important because at resting cytosolic [Ca^{2+}], most of the Ca^{2+}-binding sites of CaM are probably occupied by Mg^{2+}, which must dissociate before Ca^{2+} can bind. The C-lobe is therefore best equipped to respond rapidly to increases in cytosolic [Ca^{2+}]. Ca^{2+} binding causes twisting of the helices within the EF hand (they become almost perpendicular to each other) and, because helices from each EF hand move in concert, binding of Ca^{2+} to one EF hand promotes Ca^{2+} binding to its partner. There are also interactions between lobes and a further increase in Ca^{2+} affinity when the target protein binds; Ca^{2+} binding is thus positively cooperative (Ikura, 1996) and the affinity of CaM for Ca^{2+} is tuned by its association with target proteins (Jurado et al., 1999). These Ca^{2+}-evoked conformational changes (Fig. 1C) cause CaM to expose a concave hydrophobic surface (Vetter and Leclerc, 2003) to which target proteins can bind via positively charged amphiphilic α-helices (Rhoads and Friedberg, 1997).

Not all interactions with CaM require Ca^{2+} (Jurado et al., 1999). CaM is permanently associated with some proteins, some reversibly associate only with apoCaM, and others bind CaM whether or not it has Ca^{2+} bound. Accumulating evidence suggests that Ca^{2+}-independent anchoring of CaM to Ca^{2+}-regulated channels may be an important means of en-

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ABBREVIATIONS: CaM, calmodulin; CDF, Ca^{2+}-dependent facilitation; CDI, Ca^{2+}-dependent inactivation; IP_3R, inositol 1,4,5-trisphosphate receptor; RyR, ryanodine receptor.

suring that the Ca^{2+} sensor is placed to allow rapid responses to changes in [Ca^{2+}] (Xia et al., 1998; Liang et al., 2003; Zamponi, 2003). IQ motifs (IQxxxRGxxxR) commonly mediate Ca^{2+}-independent binding to CaM (Rhoads and Friedberg, 1997), but they are not the only sequences to mediate such interactions, nor is binding of CaM to all IQ motifs Ca^{2+}-independent (Jurado et al., 1999). An important point is exemplified by glycogen phosphorylase *b* kinase with which CaM is permanently associated: the interaction is mediated by two distinct sites in the γ subunit, neither of which binds CaM in the absence of Ca^{2+}, yet together they are sufficient to mediate a stable interaction even in the absence of Ca^{2+} (Dasgupta et al., 1989). Such synergistic binding highlights the difficulty of trying to identify physiologically important CaM-binding sites using peptide fragments of target proteins. Intimate associations between CaM and its targets tend also to be resistant to disruption by conventional or peptide antagonists (Xia et al., 1998; Krupp et al., 1999; Peterson et al., 1999). Furthermore, peptides derived from sites known to bind apoCaM in the native protein often bind preferentially to Ca^{2+}-CaM in isolation (Jurado et al., 1999). Each of these features contributes to the

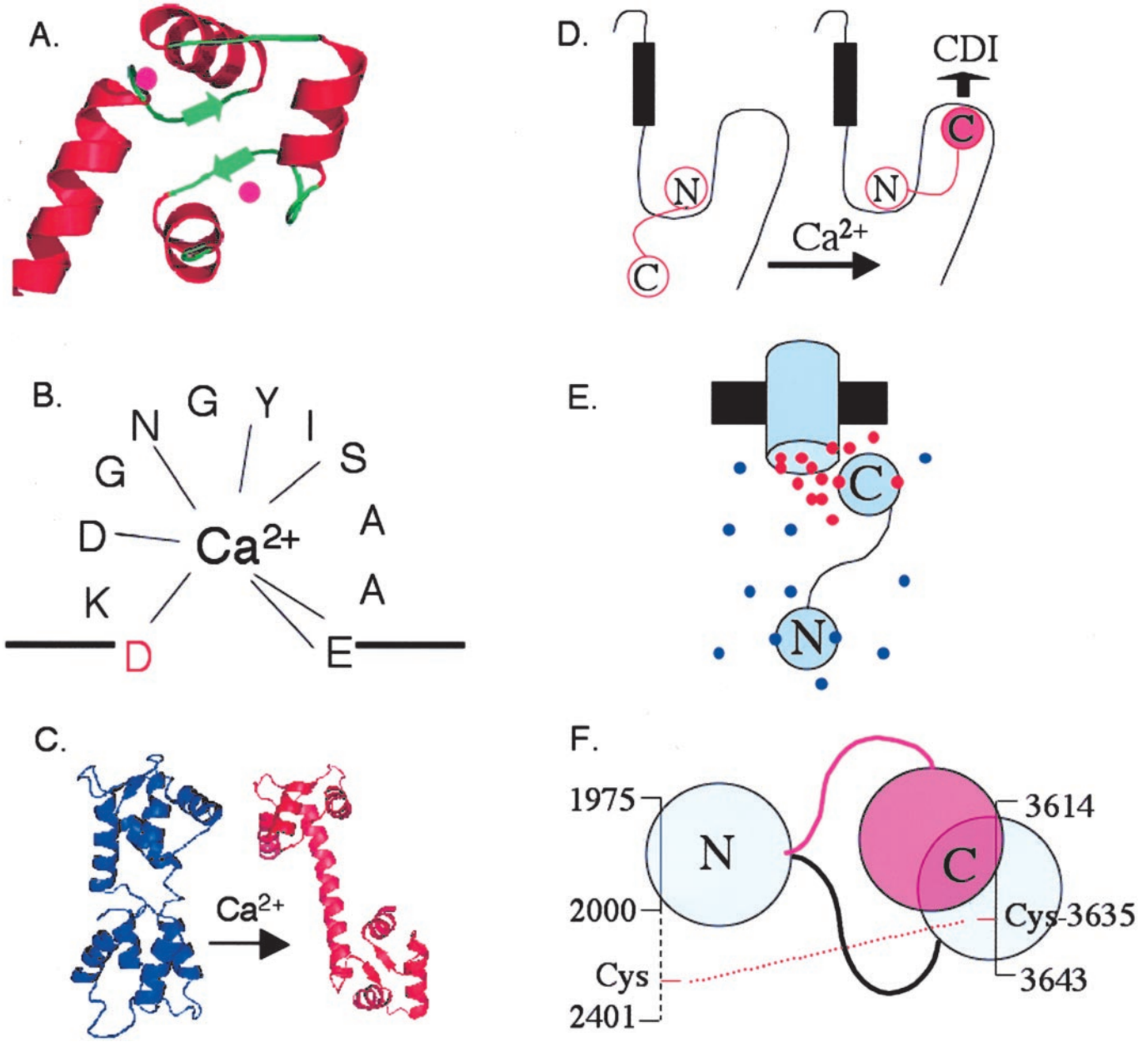

Fig. 1. A–C, Ca^{2+} binding to CaM. A, structure of the C-terminal lobe of CaM with Ca^{2+} bound (PDB code 4CLN). B, Ca^{2+}-binding loop of the third EF hand of CaM; the residue mutated to produce CaM with much reduced affinity for Ca^{2+} is shown in red. C, structures of apoCaM (blue; PDB code 1CFC) and Ca^{2+}-CaM (red; PDB code 3CLN). D–F, regulation of Ca^{2+} channels by CaM. D, CaM tethered in the C-terminal tail of the L-type Ca^{2+} channels binds Ca^{2+} and thereby acquires the ability to interact with a second CaM-binding site through which CDI is initiated. E, for non–L-type Ca^{2+} channels the two lobes of Ca^{2+} are positioned to respond to different Ca^{2+} signals, the C-lobe preferentially detects Ca^{2+} (red) passing through the channel, whereas the N-lobe responds to global Ca^{2+} signals (blue). F, in RyR1, the tethered C-lobe of CaM moves toward the N-terminal of a CaM-binding region when it binds Ca^{2+} (pink), whereas the N-lobe interacts with a site on a neighboring subunit. The C-lobe provides the essential Ca^{2+} sensor and its movement leads to channel inhibition, possibly by causing rearrangement of the interactions between the subunits. The Cys residues that also mediate cross-linking of subunits are also shown.

considerable difficulties associated with defining the roles of CaM in mediating Ca^{2+} regulation of channels.

Tethered CaM: Poised to Regulate Voltage-Gated Ca^{2+} Channels

CaM tethered to L-type Ca^{2+} channels mediates Ca^{2+}-dependent inactivation (CDI) (Peterson et al., 1999; Erickson et al., 2001). CDI is abolished in cells expressing CaM_{1234}, indicating that Ca^{2+}-insensitive CaM effectively competes with native CaM for the tethering site but fails to mediate the response to Ca^{2+}. Selective disruption of Ca^{2+} binding to either the N-lobe (CaM_{12}) or C-lobe (CaM_{34}) shows that Ca^{2+} binding to the C-lobe of CaM is required for CaM CDI. Within the C-terminal tail of the principal (α_{1C}) subunit of the L-type Ca^{2+} channel, three sequences, including an IQ motif, are capable of binding CaM (Tang et al., 2003). Exactly which of these sites contribute to CaM tethering and CDI remains controversial (Erickson et al., 2001; Pitt et al., 2001; Tang et al., 2003). Together, they mediate binding of a single CaM molecule, probably tethered by its N-lobe. Although Ca^{2+}-insensitive CaM_{1234} binds to this region, it requires basal levels of Ca^{2+}, suggesting that Ca^{2+} binding to the channel modulates its ability to tether CaM (Pitt et al., 2001). The C-lobe of CaM, when it binds Ca^{2+}, then interacts with different residues to initiate CDI. Figure 1D summarizes these interactions showing that tethered CaM migrates within the CaM-binding region when its C-lobe binds Ca^{2+} and thereby initiates CDI.

A similar mechanism underlies CDI of other voltage-gated Ca^{2+} channels but with an intriguing difference: whereas Ca^{2+} must bind to the C-lobe of CaM for inactivation of L-type channels, it binds to the N-lobe to inactivate P/Q, R, and N-type channels (Liang et al., 2003). The difference allows CDI of L-type channels to be driven by local Ca^{2+} signals (detected by the C-lobe with its ability to bind Ca^{2+} quickly; see above), whereas slower binding to the low-affinity N-lobe tailors CDI of the other Ca^{2+} channels to global Ca^{2+} signals (Fig. 1E).

For P/Q-type channels, there is an additional wrinkle. Global Ca^{2+} signals detected by the N-lobe of tethered CaM mediate CDI, whereas local Ca^{2+} signals detected by the C-lobe cause rapid Ca^{2+}-dependent facilitation (DeMaria et al., 2001). Such versatility is possible only because the two lobes of CaM respond differently to rapid increases in $[Ca^{2+}]$ and because CaM can be tethered near the mouth of the channel, where it is optimally poised both to detect and regulate channel activity. Voltage-gated Ca^{2+} channels thus provide exquisite examples of how spatial organization and the kinetics of Ca^{2+} sensors can be tailored to allow selective decoding of Ca^{2+} signals.

Tethered CaM also mediates Ca^{2+} regulation of K^+ channels (Xia et al., 1998). For other channels too, including TRP proteins, cyclic nucleotide-gated channels, and *N*-methyl-D-aspartate receptors, CaM is involved in Ca^{2+} regulation, and although the mechanisms may differ, a common theme is disruption of protein-protein interactions by Ca^{2+}-CaM.

Ca^{2+} Regulation of Intracellular Ca^{2+} Channels

Both major families of intracellular Ca^{2+} channels [i.e., inositol 1,4,5-trisphosphate receptors (IP_3R) and ryanodine receptors (RyR)] are biphasically regulated by cytosolic Ca^{2+}. Such regulation is profoundly important in that Ca^{2+} binding ultimately determines whether the channels will open: other signals exert their effects solely by modulating the effects of Ca^{2+} (Adkins and Taylor, 1999). But for neither channel is the role of CaM clearly resolved (discussed in Nadif Kasri et al., 2002; Taylor and Laude, 2002). Both channels bind apoCaM and Ca^{2+}-CaM, and CaM undoubtedly modulates their sensitivity to Ca^{2+}, but both channels also bind Ca^{2+} directly. For simplicity, we consider only Ca^{2+} inhibition.

After years during which many different CaM-binding sites were proposed to exist on RyR1, the consensus now is that each subunit binds both apoCaM and Ca^{2+}-CaM, but the sites overlap such that apoCaM is tethered by its C-lobe and then shifts within the overlapping sites when it binds Ca^{2+} (Rodney et al., 2001b; Zhang et al., 2003). The shift allows it to inhibit the channel, and CaM_{12} is as effective as wild-type CaM in mediating the inhibition (Rodney et al., 2001a). The N-lobe of CaM, which is required for inhibition even though it need not bind Ca^{2+}, seems to interact with a different site on a neighboring subunit: CaM may thereby tie one RyR1 subunit to another (Zhang et al., 2003). This is interesting because Cys residues near these CaM-binding sites also cross-link RyR1 subunits and mediate effects of oxidation on channel opening. One appealing suggestion (Xiong et al., 2002) is that CaM is tethered by both lobes and edges along its binding site when its C-lobe binds Ca^{2+}, the movement then causing rearrangement of the interaction between subunits, leading ultimately to channel inhibition (Fig. 1F).

For IP_3R, the situation is more confusing. Ca^{2+} inhibits all IP_3Rs. There is evidence that such inhibition may result from Ca^{2+} binding directly to the receptor, but competing evidence (reviewed in Taylor and Laude, 2002) implicates additional Ca^{2+}-binding proteins, and CaM is the most likely candidate (Adkins et al., 2000), not least because it has been reported to restore Ca^{2+}-inhibition to purified IP_3R (Michikawa et al., 1999). Because IP_3R1 has received most attention, we consider only this subtype. A split site near the N terminus binds both apoCaM and Ca^{2+}-CaM and seems to mediate Ca^{2+}-independent inhibition of IP_3 binding (Patel et al., 1997). It is curious, however, that CaM has never been shown to inhibit IP_3R function in the absence of Ca^{2+}. A more central site forms a typical Ca^{2+}-CaM binding site and mutation of its central Trp residue reduces its ability to bind CaM (Yamada et al., 1995). In functional assays, this mutation has consistently failed to affect inhibition of IP_3R by Ca^{2+} or CaM (Zhang and Joseph, 2001; Nosyreva et al., 2002). We have suggested that this region also binds apoCaM and that the point mutation fails to completely abolish CaM binding (Adkins et al., 2000). An article in this issue of *Molecular Pharmacology* (Nadif Kasri et al., 2004) explores this further and shows (rather like results from RyR1) that overlapping sites within this area bind both apoCaM and Ca^{2+}-CaM, such that Ca^{2+} binding might cause CaM to move toward the C-terminal of the site. The newly revealed complexity of this site re-ignites the possibility that it may contribute to CaM-regulation of IP_3R, although its absence from IP_3R3, which is also inhibited by CaM, would then be difficult to explain. A third site, which also binds only Ca^{2+}-CaM, is created only after removal of the S2 splice site of IP_3R1 (Lin et al., 2000); its role is unknown. Clearly, Ca^{2+}-CaM inhibits IP_3Rs, but

the sites through which it exerts that inhibition are unresolved, and is it unclear whether CaM is exclusively responsible for Ca^{2+} inhibition. We have suggested (Taylor and Laude, 2002; Taylor et al., 2004), in analogy with L-type Ca^{2+} channels and RyRs, that CaM may be constitutively tethered by one lobe to IP_3R1 (possibly via the N-terminal Ca^{2+}-independent CaM-binding site); then, when the second lobe binds Ca^{2+}, it binds to another site on a different subunit to inhibit channel opening. However, we need better tools to test such speculations.

At first sight, suramin, which displaces both Ca^{2+}-CaM and apoCaM from RyR1 (Papineni et al., 2002) looks like a promising means of disentangling the complex effects of CaM on IP_3R. In this issue of *Molecular Pharmacology*, Nadif-Kasri et al. (2004) show that suramin does indeed block all CaM binding to IP_3R irrespective of the free [Ca^{2+}]. However, suramin is rather like heparin in that it behaves as a competitive antagonist of IP_3; it reduces the sensitivity of Ca^{2+} release to IP_3 and it displaces IP_3 from its binding site. The authors considered this possibility, of course, and claimed to eliminate it by demonstrating that suramin effectively reduces [^{3}H]IP_3 binding to the N-terminal fragment (residues 1–581) that includes a CaM-binding site, but not to a shorter fragment (226–581). The observation is correct, but the conclusion is not. Because the shorter fragment binds IP_3 with much greater affinity (Uchiyama et al., 2002), higher concentrations of antagonist are required to displace [^{3}H]IP_3 from it. In our hands, similar fragments of IP_3R1 (1–604 and 224–604) differ by about 40-fold in their affinities for IP_3 (K_D = 5.98 ± 1.13 and 0.14 ± 0.08 nM, respectively). Suramin displaces [^{3}H]IP_3 from both, although higher concentrations are required to displace it from the smaller fragment (IC_{50} = 10.3 ± 0.6 and 129 ± 12 μM, for the long and short fragments, respectively; Fig. 2). After correcting for the different affinities for IP_3, it is clear that both fragments—one with and one without the CaM-binding site—bind suramin with similar affinity (K_D = 9.24 ± 0.6 and 20.7 ± 2.0 μM, for the long and short fragments, respectively). Plainly suramin is yet another of the low-affinity, polysulfated competitive antagonists of the IP_3R.

Despite its limitations, what can suramin tell us about the effects of CaM on IP_3R? At concentrations that prevent IP_3Rs from binding to CaM-Sepharose, suramin does not affect biphasic regulation of IP_3R by cytosolic Ca^{2+}, although the extent of the inhibition was surprisingly small. This might suggest that CaM cannot provide the Ca^{2+} sensor that mediates Ca^{2+} inhibition, but it is notoriously difficult (see above) to pharmacologically disrupt the association of endogenous CaM with its targets. In none of these experiments was suramin shown to dissociate endogenous CaM from full-length IP_3Rs. More interesting is the observation that CaM_{1234} effectively mimics the effects of CaM: both potentiate Ca^{2+} inhibition (Nadif Kasri et al., 2004). This surprising result is very different from the effect of CaM_{1234} on other Ca^{2+}-regulated channels, where it behaves as an antagonist by displacing endogenous Ca^{2+}-responsive CaM. For IP_3R1, then, the effects of CaM are wholly independent of its ability to bind Ca^{2+}, but the CaM can inhibit only when the IP_3R has bound Ca^{2+}. Many sites to which Ca^{2+} binds on IP_3R1 (Sienaert et al., 1997) could mediate the effect of Ca^{2+}, but where does the CaM bind? The N-terminal CaM-binding site deserves consideration, but in the intact IP_3R, both CaM binding to it (unlike the "apoCaM"-binding site of L-type channels) and the effect of CaM on IP_3 binding are entirely unaffected by Ca^{2+} (Patel et al., 1997). This site might therefore be responsible for CaM tethering, but it seems unlikely to mediate a response to Ca^{2+}. Perhaps, in analogy with the behavior of CaM at other Ca^{2+}-regulated channels, the work from Nadif Kasri et al. should prompt us into looking for another apoCaM-binding site, access to which is regulated by Ca^{2+} binding to the IP_3R.

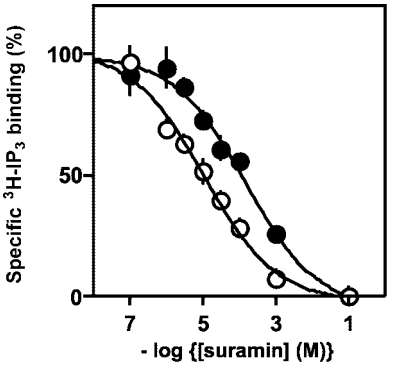

Fig. 2. Binding of suramin to N-terminal fragments of IP_3R1. Displacement of [^{3}H]IP_3 (0.7 nM) from N-terminal fragments (●, residues 224–604; ○, 1–604) of IP_3R1 by suramin (means ± S.E.M, n = 3).

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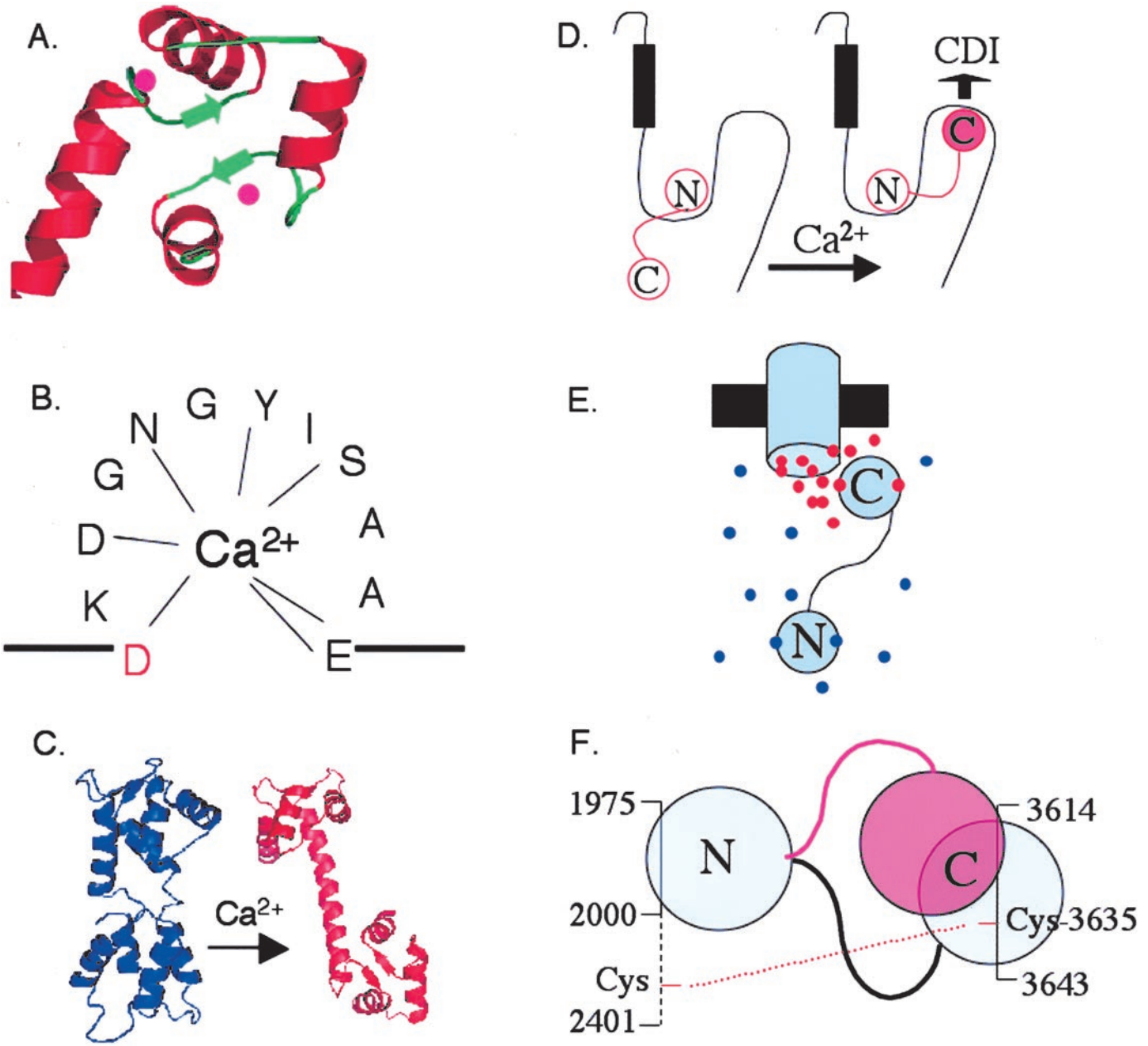
Correction to "Ca^{2+} regulation of inositol 1,4,5-trisphosphate receptors: can Ca^{2+} function without calmodulin?"

In the above article [Rossi AM and Taylor CW (2004) *Mol Pharmacol* **66:**199–203], Fig. 1 was inadvertently printed in black white. The color figure appears below. The online version has been corrected in departure from the print version.

We regret this error and apologize for any confusion or inconvenience it may have caused.



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