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Feedback inhibition of Ca^{2+} channels by Ca^{2+} depends on a short sequence of the C terminus that does not include the Ca^{2+}-binding function of a motif with similarity to Ca^{2+}-binding domains

(heart/neurons/EF hands/chimeras)

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ABSTRACT **α_{1C}- and α_{1E}-based Ca^{2+} channels differ in that the former are inhibited by Ca^{2+} entering through its pore, while the latter are not. It has been proposed on the basis of analysis of α_{1E}/α_{1C} chimeras that the molecular determinants responsible for Ca^{2+} inhibition involve both a conserved Ca^{2+}-binding motif (EF hand) plus additional sequences located C-terminal to the EF hand. Through construction of similar α_{1E}/α_{1C} chimeras, we transferred Ca^{2+} inhibition from α_{1C} to α_{1E} by replacing a 134-aa segment of α_{1E} with the homologous 142-aa segment of α_{1C}. This segment is located immediately after the proposed Ca^{2+}-binding EF hand motif. Replacement of the α_{1C} EF hand with the corresponding EF hand of α_{1E} did not interfere with inhibition of α_{1C} by Ca^{2+}, and a triple mutant of α_{1C}, α_{1C}[D1535A,E1537A,D1546A], that disrupts the potential Ca^{2+}-coordinating ability of the EF hand continued to be inhibited by Ca^{2+}. These results indicate that a small portion of the α_{1C} C terminus is essential for inhibition by Ca^{2+} and place the Ca^{2+}-binding site anywhere in α_{1C}, with the exception of its EF hand-like motif.**

Ca^{2+} entry into cells through voltage-activated Ca^{2+} channels is transient as a result of voltage-induced inactivation and/or because of feedback inhibition by Ca^{2+} itself. We reported previously that, in *Xenopus* oocytes, concentrations of 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate (BAPTA) that are able to block activation of Ca^{2+}-activated Cl^- currents by Ca^{2+} entering through the mouth of the Ca^{2+} channel do not inhibit Ca^{2+}-dependent inhibition of the channel (1) and concluded that the Ca^{2+}-binding site should be very close to the conduction pathway. But its actual location remained unknown. Biochemical studies and molecular cloning have shown that Ca^{2+} channels are formed of an α_1 subunit and accessory regulatory subunits. Some α_1 subunits—e.g., α_{1C}—are feedback-inhibited by Ca^{2+} (1), while others—e.g., α_{1E}—are feedback-regulated only by voltage (2). Recently, de Leon *et al.* (3) reported that they were able to confer Ca^{2+} inhibition to α_{1E} by replacing the entire amino acid C terminus of α_{1E} with a 217-aa truncated form of the α_{1C} C terminus. This segment includes a 29-aa motif with homology to classical Ca^{2+}-binding domains called EF hands (4). This motif thus was proposed by de Leon *et al.* (3) to be the Ca^{2+}-binding site responsible for channel inhibition. We have also been studying the structural basis for Ca^{2+} inhibition, and we have also found this general region of α_{1C} to be essential for feedback inhibition by Ca^{2+}. However, as we report below, it excludes both the EF hand and a Ca^{2+}-binding function that may be associated with it.

METHODS

Construction of α_{1C}/α_{1E} Chimeras and Mutants. To facilitate the engineering of α_{1C} and α_{1E} cDNAs, we subjected both cDNAs to silent mutagenesis to remove undesirable restriction sites and introduce new ones without altering the amino acid sequence encoded in the cDNAs. For α_{1C}, we used the DN60 derivative of clone Va.33 (ref. 5; GenBank accession no. X15539) in which amino acids 1–59 have been removed and eliminated the restriction sites *Ssp*I at nucleotide 1970, *Sac*II at nucleotide 4116, and *Sca*I at nucleotide 4969, and we created sites for *Bst*BI at nucleotide 465, *Sca*I at nucleotide 1288, *Hpa*I at nucleotide 1745, *Sal*I at nucleotide 2348, *Sac*II at nucleotide 2845, *Ssp*I at nucleotide 3569, and *Bst*BI at nucleotide 3752. Va.61 is the engineered α_{1C} cDNA clone that resulted from these manipulations. For α_{1E}, we removed from clone E239 (ref. 2; GenBank accession no. L27745 with the 5′ extension shown below) the restriction sites *Kpn*I at nucleotide 658, *Bst*BI at nucleotide 1231, *Ssp*I at nucleotide 1736, and *Bst*BI at nucleotide 6753, and we created sites for *Hpa*I at nucleotide 1510, *Sal*I at nucleotide 2109, *Sac*II at nucleotide 3505, *Ssp*I at nucleotide 4247, *Bst*BI at nucleotide 4436, and *Mlu*I at nucleotide 5181. E101 is the engineered α_{1E} cDNA clone that resulted from these manipulations. α_{1E}/α_{1C} chimeras (EC chimeras) and mutants/deletions were made by standard two-round PCR or M13-based site-directed mutagenesis (6), using Va.61 and E101 as substrates. The nucleotide sequence of all modified cDNAs was confirmed by the dideoxy chain termination method of Sanger *et al.* (7) using the double-stranded plasmids as templates. The open reading frames of Va.61 and E101 and their derivatives were placed into a modified pAGA2 vector, which is a pGEM3-based plasmid (5). E101 and its derivatives were placed into pGEM3 downstream of the T7 promoter so that the nucleotide sequence between the T7 promoter and the initiator ATG in L27745 became 5′-GGGAGACCGG AATTGATCCC CGGGTACCAT GGTGTGTCTT CTGTCTGTTT AAACCTCAGG **ATG**, where the **A** of **ATG** corresponds to nucleotide 1 of the open reading frame reported in L27745; the nucleotide sequence

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Abbreviation: BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate.

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between the stop codon and the beginning of the poly(A) tail of pAGA2 is 5′-**TAG** AGGCTGCTCC CCCCTCCGAT GCATGCTCTT CTCTCACATG GAGAAAACCA AGACAGAATT GGGAAGCCAG TGCGGCCCGG GGGGGAGGAA GAGGAAGAGG GAAAAGTCGT CCTGTTGTAG GCCTCCCCCT AGCATCCTCT TAG, where **TAG** is the stop codon of the open reading frame. For Va.61 the nucleotide sequence between the T7 promoter and the initiator ATG in X15539 is 5′-TAATACGACT CACTATAGGG AGACCGGAAT TGATCCCCGG GTACC **ATG**, and the sequence between the stop codon and the beginning of the poly(A) tail of pAGA2 is 5′-**TGA** GCGCCAGGGC CGGGGGTGCG GGTTTTTTAT TTGTCTCAAT GTTCCTAATG GGTTCGTTTC AGAACGTTTC AGAAGTGCCT CACTGTTCTC GTGACCTGGA GTTAACCGCG GAATTGGGAT CCTCTAGCTA G.

The rat β2a subunit (ref. 8; GenBank accession no. M80545) was cloned as a *Nco*I/*Xba*I fragment of clone βb24 into *Nco*I/*Xba*I-digested pAGA2 by changing codon 2 from CAG (Q) to GAG (E) and adding a TCTAGA *Xba*I restriction site immediately after the TGA stop codon.

Synthesis of cRNAs. *Hin*dIII-digested DNA templates (1 μg) were transcribed in a final volume of 20 μl with reagents provided in the mMesSAGE mMACHINE cRNA synthesis kit from Ambion (Austin, TX; catalog no. 1344). After removal of the template DNA by treatment with RNase-free DNase I and precipitation with either LiCl or ammonium acetate, the cRNAs were dissolved in double-distilled diethyl-pyrocarbonate (DEPC)-treated water to a final concentration of 1–2 μg/μl. Wild-type or mutant α_1 subunits were expressed in *Xenopus* oocytes together with the rat β2a subunit. To this end, cRNAs encoding wild-type or mutant α_{1C}, wild-type or mutant α_{1E}, or α_{1E}/α_{1C} chimeras (0.2 μg/μl) were coinjected into oocytes with 0.2 μg/μl of rat β2a cRNA.

Oocyte Preparation. Frogs (*Xenopus laevis*) were anesthetized by immersion into 0.15–0.17% tricaine methanesulfonate in water and removed from the tricaine methanesulfonate bath. Ovarian lobes were then exposed through a small incision made into their abdominal wall, removed, and placed into sterilized Ca^{2+}-free OR-2 solution (82.5 mM NaCl/2.5 mM KCl/1 mM $MgCl_2$/5 mM Hepes, pH adjusted to 7.6 with NaOH), and the frogs were returned to tricaine-free water for recovery. The ovarian lobes were then rinsed with sterile water, teased open, and incubated at room temperature in Ca^{2+}-free OR-2 containing 2 mg/ml collagenase (type I; BRL) to cause release and defolliculation of oocytes. After 1 hr on an orbital shaker ($\approx$60 cycles per min), the oocytes were transferred to a Petri dish with OR-2. Dead and too-small oocytes were removed by aspiration, and the selected oocytes were washed several times with collagenase-free and Ca^{2+}-free OR-2 solution, incubated under agitation for an additional 1 hr with solution changes every 7–8 min, and placed into an incubator at 19°C and incubated for an additional 1 hr in a 1:4 mixture of sterile SOS (100 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes, pH adjusted to 7.6 with NaOH) and Ca^{2+}-free OR-2 solutions and 30 min in 1:3 SOS/Ca^{2+}-free OR-2. The oocytes were then placed into 100% SOS and selected once more, by removing those that are dead or too small and kept at 19°C until injected.

Injected oocytes were kept at 17–19°C, with daily solution changes in sterile SOS containing 50 μg/ml gentamycin, until they were used for electrophysiological testing (4–6 days).

Electrophysiological Recordings of Ca^{2+} Channel Currents from Oocytes. The cut-open vaseline gap voltage-clamp method (9) as modified (1, 10) was used throughout. Activation of Cl^- current by Ba^{2+} or Ca^{2+} influx through the Ca^{2+} channel was eliminated by injecting 100–150 nl of 50 mM Na_4BAPTA before recording (1). The BAPTA solution was adjusted to pH 7.0 with methanesulfonic acid. Ba^{2+} currents were recorded using an external solution containing 10 mM Ba^{2+}, 96 mM Na^+, 0.1 mM ouabain, and 10 mM Hepes, titrated to pH 7.0 with methanesulfonic acid (CH_3SO_3H). Unless indicated otherwise, Ca^{2+} currents were recorded with the same external solution but replacing Ba^{2+} with 10 mM Ca^{2+}. The solution in contact with the oocyte interior was 110 mM K-glutamate/10 mM Hepes, titrated to pH 7.0 with KOH. Low access resistance to the oocyte interior was obtained by permeabilizing the oocyte with 0.1% saponin. The holding potential was -90 mV. The linear components of the currents, corresponding to the scaled currents elicited by small negative control pulses of one-quarter the amplitude of the stimulating pulse, were subtracted online. Data were collected at a sampling frequency of 1 kHz and filtered at 200 Hz.

Presentation of Results. Inactivation rates ($1/\tau$) in the presence of Ca^{2+} (*ir*Ca) or Ba^{2+} (*ir*Ba) were obtained from least squares fits of the first 350–1000 msec of current inactivation time courses to a first-order decay function ($A/A_o = \exp(-t/\tau) + C$). For certain α_{1E}/α_{1C} chimeras, channel inactivation is purely voltage-dependent when measured in external Ba^{2+}, but it is the sum of coexisting voltage-dependent and Ca^{2+}-dependent processes when measured in external Ca^{2+}. Where each of these these effects were very marked—e.g., chimeras EC1 and EC56—we desisted from calculating *ir*Ca, as we could not define in the chimera the proportion of inactivation that was due to Ca^{2+}. For these chimeras we thus present only the direct data to indicate whether or not a Ca^{2+} inactivation component has been introduced into the α_{1E}.

RESULTS AND DISCUSSION

To study Ca^{2+} channel inhibition by Ca^{2+}, we expressed α_{1C}, α_{1E}, or α_{1C}/α_{1E} chimeras in *Xenopus* oocytes together with β_{2a}. A β subunit was coexpressed to take advantage of the action of this regulatory subunit to improve the coupling between voltage sensing and pore opening, which lead to an increase in the magnitude of the α_{1C} currents (10). The 2a subtype of β was chosen to take advantage of its effect to delay and reduce voltage-induced inactivation of α_{1E} (11) and thus alleviate the effect of this complicating feature in the analysis of an inactivating effect of Ca^{2+} in α_{1E}/α_{1C} chimeras. For construction of chimeras, we chose as crossover points sites in the amino acid sequence at which α_{1C} and α_{1E} were identical. The rationale behind this choice was the assumption that the two molecules were not only homologous at the level of linear amino acid sequence composition but also at the level of their three-dimensional structure, so that identical amino acids in the linear sequence could be expected to adopt similar orientations in space. This should then allow the exchange of structures spanned by similarly placed amino acids and minimize folding problems of the chimera. In spite of this, chimera EC58 was not expressed, indicating that additional factors influence the functional expression of a membrane protein.

Assessment of Inhibition by Ca^{2+}. Results reported previously had shown that the site to which Ca^{2+} binds to inhibit α_{1C} channel activity could be located very close to the inner mouth of the channel, as injection into oocytes of the Ca^{2+} chelator BAPTA was able to interfere with activation of Cl^- currents by Ca^{2+} entering through the channel's pore but not with the inactivation of the channel by the incoming Ca^{2+} (1). As a consequence, α_{1C} inhibition by Ca^{2+} varies with voltage. It is minimal at low test potentials at which single channel amplitudes are large, mean open times are short, and mean closed times are long. Only minimal Ca^{2+} influx as a function of time occurs under these conditions. Failure of Ca^{2+} inhibition at low test potentials could then be due either to failure of Ca^{2+} binding to the inhibitory site or, because of dissociation during the time the channel is closed and attendant failure in occupying the inhibitory site by Ca^{2+} for a sufficiently long time to allow for transduction of the binding signal into the conformational cascade responsible for inactivation. Inactivation is

also minimal at test potentials close to the reversal potential, at which single channel amplitudes are near zero, even though mean open times are a maximum. Inhibition is maximal at voltages close to those eliciting maximal macroscopic Ca^{2+} currents, at which influx of Ca^{2+} per unit time is maximal (or close to it). For reasons that are still under study, a typical characteristic of the rate of inhibition by Ca^{2+} is that it is maximal at voltages slightly below those at which current–voltage relations show their maxima (Fig. 1).

Structural Determinant Conferring Ca^{2+} Inhibition. Point mutations and amino acid deletions may have long-range effects on the conformation and function of a protein and therefore do not necessarily pinpoint location of binding and/or regulatory sites. Because of this, we sought to locate the region(s) of cardiac α_{1C} responsible for feedback inhibition by Ca^{2+} through a gain-of-function approach in which we replaced segments of the Ca^{2+}-unresponsive α_{1E} with the corresponding and structurally homologous segments of the Ca^{2+}-responsive α_{1C}. Fig. 2 depicts the strategy used and a summary of the results obtained with different EC chimeras and α_{1E} and α_{1C} mutants. The patterns of inactivation as a function of test potentials obtained in Ba^{2+} vs. those obtained in Ca^{2+} for EC1, α_{1E} with the C terminus of α_{1C}, and EC 56, α_{1E} with the 142-aa RL → VS segment from α_{1C}, are shown in Fig. 3. Those of $\alpha_{1C}[EF_E]$, α_{1C} with the EF hand from α_{1E}, are shown in Fig. 4. The results indicate that inhibition by Ca^{2+} can be conferred to α_{1E} by replacing of a small segment of the C-terminal tail of α_{1E} with the corresponding segment of α_{1C} and that the α_{1E} EF hand can substitute for that of α_{1C} in α_{1C}.

Lack of a Ca^{2+}-Binding Role for the EF Hand Motif of α_{1C}. The results on Ca^{2+} sensitivity of α_{1C}/α_{1E} chimeras shown in Figs. 3 and 4 *A* and *B* agree only partially with those reported on similar chimeras by de Leon *et al.* (3). On one hand, we confirmed that Ca^{2+} inhibition is encoded in the C terminus of α_{1C}, because replacing both the EF-like motif plus the C-terminal extension of α_{1E} with the corresponding segment of α_{1C}, resulted in appearance of inhibition by Ca^{2+} in an α_{1E} channel (Fig. 3 *Upper*). On the other hand, unlike the findings of de Leon *et al.* (3), our findings showed that an α_{1C} in which its EF-like motif is replaced with the corresponding segment from α_{1E} is still inhibited by Ca^{2+} (Fig. 4 *A* and *B*). Since this α_{1E} segment contains an EF-like motif that is as similar to consensus EF hands as that of the α_{1C} it replaced (Fig. 5), this result can be interpreted in two ways. One assumes that neither the α_{1C} nor the α_{1E} EF hand-like motifs bind Ca^{2+}. The results would then pinpoint the RL → VS segment as the critical determinant required for establishment of Ca^{2+} inhibition and leave open the question of where Ca^{2+} binds to exert its action.

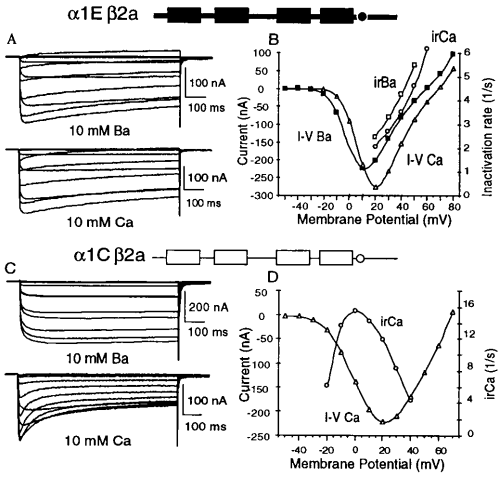

FIG. 1. Inhibition by Ca^{2+} of α_{1C} but not α_{1E} and comparison of voltage dependence of ir_{CA} to that of the current.

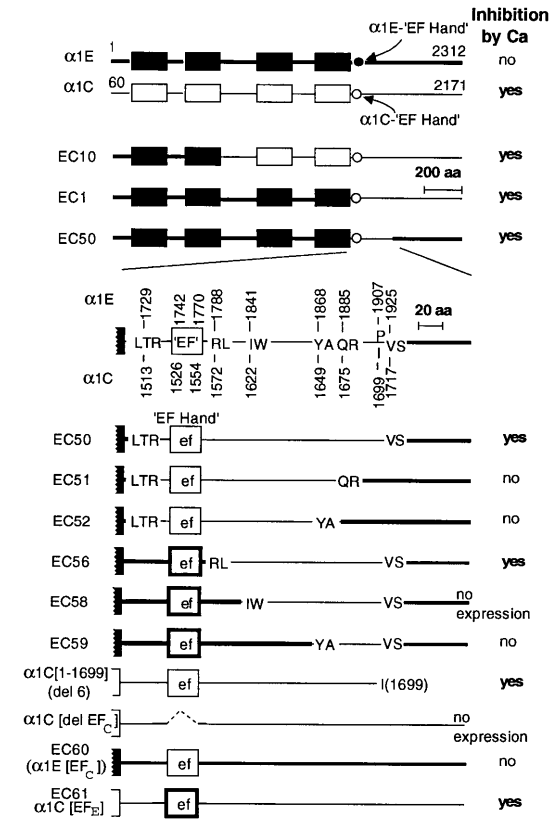

FIG. 2. Schematic representation of α_{1E}/α_{1C} chimeras and key results obtained. EC1, $\alpha_{1E}1728/\alpha_{1C}(1513–2171)$; EC10, $\alpha_{1E}(1–703)/\alpha_{1C}(784–2171)$; EC50, $\alpha_{1E}(1–1728)/\alpha_{1C}(1513–1717)/\alpha_{1E}(1926–2312)$; EC60, $\alpha_{1E}(1–1741)/\alpha_{1C}(1526–1554)/\alpha_{1E}(1771–2312)$; and EC61, $\alpha_{1C}(60–1525)/\alpha_{1E}(1742–1770)/\alpha_{1C}(1555–2171)$. Amino acid sequences LTR, RL, IW, YA, QR, and VS were common to α_{1C} and α_{1E} and were used as crossover points in the construction of chimeras. Deletion of the EF hand sequence from α_{1C} impeded expression of the protein on the oocyte surface, as neither ionic nor gating currents were detected.

The second assumes that both EF hand-like motifs have the ability to bind Ca^{2+} if they are supported by the proper sequence environment and that, in this case, this environment is provided by the RL → VS segment of the α_{1C}. Except for the fact that the α_{1E} EF hand motif was inactive in the context of α_{1C}, this was the general conclusion drawn by de Leon *et al.* (3).

To distinguish between the two possibilities listed in the previous paragraph, we tested validity of the second—i.e., whether the EF-like motif of the Ca^{2+}-sensitive α_{1C} plays a Ca^{2+}-binding role. If so, disruption of the Ca^{2+}-binding ability

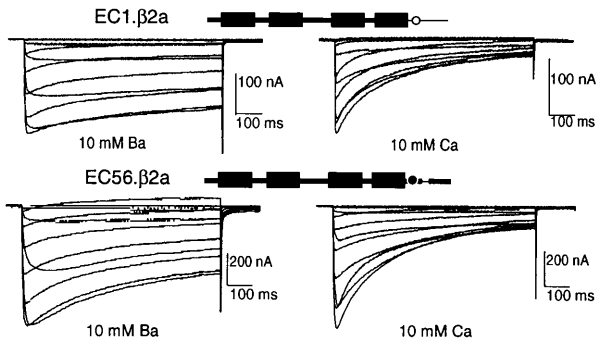

FIG. 3. Chimeras EC1 (α_{1E} with C terminus of α_{1C}) and EC56 (α_{1E} with RL-VS segment of α_{1C}) are inhibited by Ca^{2+}. Ca^{2+} inhibition of the parental α_{1C} and lack thereof in the parental α_{1E} are shown in Fig. 1.

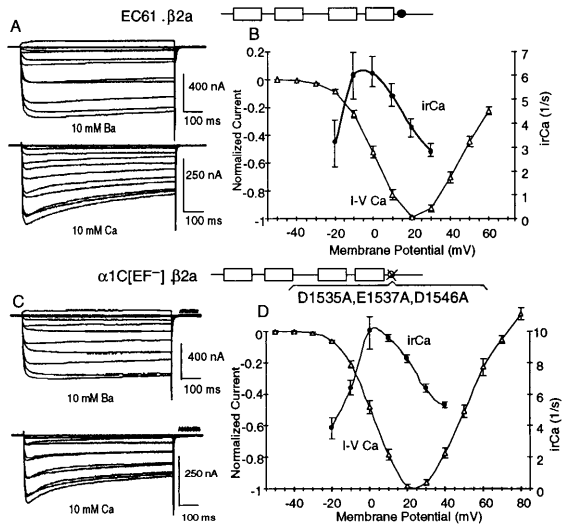

FIG. 4. EC61, α_{1C} with the EF motif of α_{1E}, and α_{1C}[[D1535A,E1537A,D1546A], an α_{1C} lacking a functional EF motif, are both subject to Ca^{2+} inhibition.

should interfere with inhibition by Ca^{2+}. We thus introduced "inactivating" mutations into the EF-like motif of α_{1C}.

Ca^{2+}-binding EF hands have been extensively studied by x-ray crystallography and nuclear magnetic resonance (for review see ref. 13). In all cases Ca^{2+} has been found to be held by seven coordination bonds that define an octahedron with vertices $X, Y, Z, -X, -Y$, and $-Z$ (Fig. 5*A*), of which vertex $-Z$ is bidentate. The coordinating oxygens at $-Z$ are most frequently the two ε oxygens of a Glu extending from the F helix, but can also be the two δ oxygens of an Asp extending from the same location in helix F. The coordinating atom at $-Y$ is always a carbonyl oxygen of the peptide backbone that forms the loop connecting the E and F helices, making the side chain at this position irrelevant. In most but not all of the EF hands with bound Ca^{2+} described thus far, the coordinating oxygen at $-X$ is provided by H_2O (W in Fig. 5), allowing this residue to be almost any amino acid. The coordinating atoms at positions $-X, X$, and Z are most frequently a δ oxygen of Asp or Asn or one of the ε oxygens of Glu. The coordinating atom at position X has also been found to be the γ oxygen of Ser or Thr or the γ sulfur of Cys (cf. ref. 13). A two-dimensional scheme of a typical EF hand with the corresponding assignments of the coordination bonds is shown for site 4 of chicken troponin C in Fig. 5*A Right*. The equivalent diagram, predicted by computerized sequence analysis of α_{1C} (4) and α_{1E} (2), shows that the α_{1E} and α_{1C} EF hands are potentially capable of satisfying six of the seven Ca^{2+} coordination bonds provided by a bona fide Ca^{2+}-binding EF hand (Fig. 5*B*). It needs to be emphasized, however, that even if all seven coordination bonds can be satisfied by sequence similarity analysis, this by no means proves that the EF hand modeled in this way will indeed bind Ca^{2+}. This would require additional knowledge, including the relative orientations of the E and F helices, and hence the spacial disposition of the residues that provide the atoms that coordinate the Ca^{2+}. Thus, Ca^{2+} does not bind to site 1 of chicken troponin C and of the four EF hands of recoverin, only one is occupied by Ca^{2+} (14), even though these sites are predicted to bind Ca^{2+} by the type of analysis that has predicted the α_{1C} and α_{1E} EF hands.

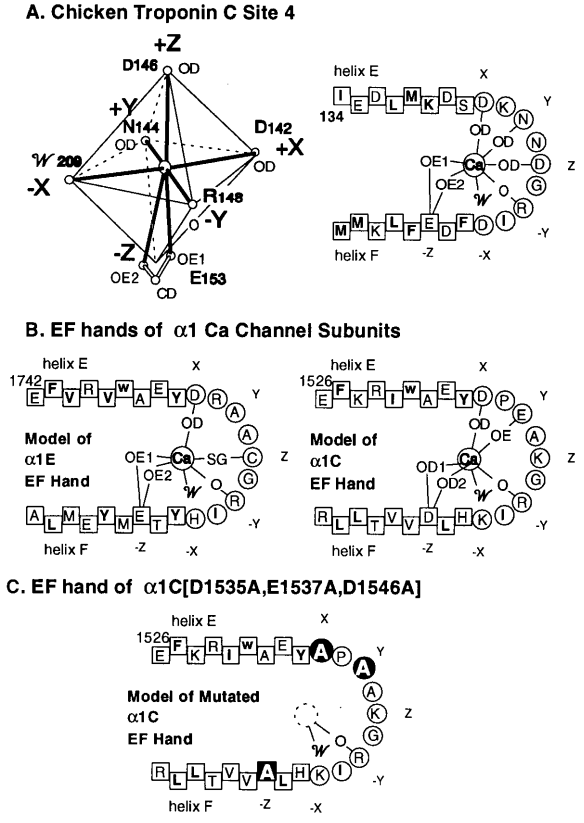

FIG. 5. Ca^{2+} coordination octahedron in a typical EF hand (*A Left*); its two-dimensional projection (*A Right*), and the predicted coordination of Ca^{2+} by the EF hands of α_{1E} (*B Left*), α_{1C} (*B Right*), and α_{1C}[[D1535A,E1537A,D1546A] (*C*). Ca^{2+} binding to site 4 of chicken troponin C (cTnC) is adapted from Satyshur *et al.* (12). O, carbonyl oxygen of the peptide bond; 0D, δ oxygen of Asp or Asn; OE, ε oxygen of Glu; and $\mathcal{W}$, water. Amino acids in squares are part of either the E or the F helices of the (postulated) EF hand; amino acids in circles form the connecting loop of the helix–loop–helix structure. The amino acids that contribute to the formation of the octahedral Ca^{2+}-coordinating cage are labeled $X, Y, Z, -X, -Y$, and $-Z$.

Fig. 5*C* illustrates the triple-inactivating mutation introduced into α_{1C} (i.e., the change of Asp, Glu, and Asp at positions X, Y, and $-Z$ of the α_{1C} EF motif to Ala), creating α_{1C}[D1535A,E1537A,D1546A]. This triple change is predicted to render the putative Ca^{2+}-binding motif incapable of binding Ca^{2+}. Involvement of a Ca^{2+}-binding function at the EF motif would then be predicted to render the mutant channel insensitive to Ca^{2+}. We found however, that α_{1C}[D1535A,E1537A,D1546A] is inhibited by Ca^{2+} (Fig. 4 *C* and *D*).

Taken together, these results indicate that neither α_{1E} nor α_{1C} has an operational Ca^{2+}-binding EF hand and that Ca^{2+} inhibition can be conferred to an α_1 by introduction of a segment of the α_{1C} C terminus. A likely interpretation of the data is therefore that inhibition by Ca^{2+} is due to binding to an as yet undefined site on α_1. Based on our previous finding that Ca^{2+} inhibition of α_{1C} is not interfered with by injection of up to 100 nl of 100 mM BAPTA, which is sufficient to suppress Ca^{2+}-activated Cl^- currents (1), the inhibitory site for Ca^{2+} should be very close to the internal mouth of the channel. Results from Eckert and Tillotson (15) and Imredy and Yue (16), showing blockade of inhibition by Ca^{2+} by chelation of Ca^{2+} in cells that naturally express Ca^{2+} channels, place the Ca^{2+}-binding site outside the conduction path proper. In as yet unreported experiments, instead of injecting BAPTA, we perfused oocytes expressing $\alpha_{1C}\beta 2a$ with 500 μM BAPTA and observed first unmasking of the Ca^{2+} inhibition, as Cl^- currents were suppressed, and then its progressive suppression, indicating that also for the Ca^{2+} channel complex expressed in the oocyte, the Ca^{2+}-binding site is outside of the conduction pathway (F.N., R.O., and E.S., unpublished work). Further studies are needed to determine the exact location of the Ca^{2+}-binding site and the mechanism by which Ca^{2+} inactivates the channel in the manner it does.

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