Ca²⁺ sensors of L-type Ca²⁺ channel

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Abstract Ca²⁺-induced inactivation of L-type Ca²⁺ is differentially mediated by two C-terminal motifs of the α_{1C} subunit, L (1572–1587) and K (1599–1651) implicated for calmodulin binding. We found that motif L is composed of a highly selective Ca²⁺ sensor and an adjacent Ca²⁺-independent tethering site for calmodulin. The Ca²⁺ sensor contributes to higher Ca²⁺ sensitivity of the motif L complex with calmodulin. Since only combined mutation of both sites removes Ca²⁺-dependent current decay, the two-site modulation by Ca²⁺ and calmodulin may underlie Ca²⁺-induced inactivation of the channel. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Heart; Calcium channel; Calmodulin; Ca^{2+} sensor; Inactivation

1. Introduction

The voltage-gated L-type Ca²⁺ channel is an oligomeric complex composed of the pore-forming α_{1C} and auxiliary β and α 2-SS- δ subunits [1]. Similar to the channel initially described in *Paramecium* [2], the α_{1C} channel is inhibited by Ca²⁺ but not by Ba²⁺ ions on the cytoplasmic side of the pore [3–6]. This Ca²⁺-induced inactivation serves as an important feedback mechanism against Ca²⁺ overloading of the cell.

The α_{1C} subunit is built of over 50 exons [7]. Alternative splicing of exons 40–42 causing substitution of 80 amino acids (1572–1651) in the C-terminal tail of the conventional $\alpha_{1C,77}$ channel [8] for 81 different amino acids deprives the channel of Ca²⁺-induced inactivation [9]. Within these 80 amino acids there are two sequences, L (1572–1587) and K (1599–1651), independently critical for Ca²⁺-induced inactivation [10]. A calmodulin (CaM)¹ binding IQ-like region was proposed within the sequence K [11]. The role of CaM as important mediator of Ca²⁺-induced inactivation of the channel and its binding in the presence of Ca²⁺ to the IQ-like peptide were later confirmed [12–14]. However, the mechanism of Ca²⁺-induced inactivation remains unclear in part because it is not understood (i) whether motif L is involved in CaM-mediated regulation of inactivation, (ii) the location or role of

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CaM in the resting state of the channel when the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) is critically low for the occupancy of the IQ region, and (iii) how the rise in $[Ca^{2+}]_i$ is transduced by CaM into inactivation of the channel.

In this study we found that motif L serves both as a Ca^{2+} sensor and CaM-binding site at low resting $[Ca^{2+}]$. Increase in $[Ca^{2+}]$ causes conformational rearrangement in the sensor which is apparently transduced to higher affinity of CaM interaction with motif L. We conclude that Ca^{2+} -dependent transition in the Ca^{2+} sensor of motif L may underlie CaM-mediated Ca^{2+} -induced inactivation of the channel.

2. Materials and methods

2.1. DNA constructs

Kozak sequence (5'-CCGCCA-3') was incorporated at *Hin*dIII/ *NcoI* sites of pHLCC77 [8] using the mixture of phosphorylated oligonucleotides 5'-AGCTTGGATCCGCCAC-3' and 5'-CATGGTG-GCGGATCCA-3', and with the full coding sequence it was subcloned into pcDNA3 vector (Invitrogen). Mutated Ca²⁺ channel plasmid 77LpcDNA3 was prepared by ligating the 3.9-kb *Bam*HI(blunt)/*Ppu*-MI fragment of pHLCC77L [10] with *Ppu*MI/*Not*I(blunt) fragment of 77pcDNA3. CaM_{WT}pcDNA3 and CaM_QpcDNA3 were prepared by ligating the *SaII*(blunt)/*BgIII* fragments of CaM_{WT}-pBF and CaM_QpBF [15], respectively, at *Hin*dIII(blunt)/*Bam*HI sites of pcDNA3. α_{1Ca} , β_{2A} and $\alpha_{2}\delta$ in pcDNA3 were gifts of F. Hofmann [16].

2.2. Cell transfection and electrophysiological recording

30–50% confluent tsA201 cells were transfected with 2.5 µg of total cDNA (molar ratio of α_{1C} : β_{2A} : $\alpha_2\delta$:CaM:EGFP:CD8 = 1:1.6:1.4: 4:2.5:0.7) using SuperFect (Qiagen). Transfection efficiency was estimated by EGFP fluorescence to be $\approx 20-60\%$. Co-expression of CD8 receptor was used to visually identify expressing cells for electrophysiological experiments by binding of anti-CD8 antibody-coated magnetic beads (Dynal). The whole-cell patch clamp bath solution contained (mM): 125 *N*-methylglucamine, 1 MgCl₂, 10 D-glucose, 10 HEPES, 5 CsCl, 20 BaCl₂ or CaCl₂. The pipet solution contained (mM): 60 CsCl, 1 CaCl₂, 11 EGTA, 10 HEPES, 50 aspartic acid, 5 MgATP, pH 7.4 (CsOH). Maximum activation of the $\alpha_{1C,77}$ and $\alpha_{1C,77L}$ channel currents was obtained by depolarizations to 20/20 mV and 20/30 mV with Ba²⁺/Ca²⁺ as charge carrier, respectively.

2.3. Glutathione S-transferase (GST) pulldown

GST- $\alpha_{1C(1414-1649)}$ fusion protein was expressed using the *Eco*RV (4541)/*FspI* (5247) fragment of α_{1Ca} subcloned into pGEX vector (Pharmacia). GST- $\alpha_{1C(1414-1649)}$ was immobilized on glutathione–Sepharose, incubated with CaM for 2 h in 150 mM NaCl, 50 mM Tris–HCl, pH 8.0 containing 100 μ M Ca²⁺ or 2 mM EGTA, and washed with the same buffer containing 0.05% Tween-20. Bound proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) (12%) and identified by Western blot analysis with anti-CaM (Upstate Biotechnology) or anti-GST antibodies (Pharmacia).

2.4. Peptide–CaM binding PAGE shift assay

300 pmol of hog brain CaM (Pharmacia) was incubated for 30 min

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Abbreviations: CaM, calmodulin; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis



Fig. 1. Ca²⁺-dependent and Ca²⁺-independent binding of CaM to the α_{1C} C-terminal tail. Shown is the GST pulldown assay of CaM binding to GST- $\alpha_{1C(1414-1649)}$ fusion protein in 100 μ M [Ca²⁺] (left) or 2 mM EGTA (right). No binding of CaM to GST was detected under these conditions. Molecular weights (left) and positions (right, marked by arrows) of CaM and GST- $\alpha_{1C(1414-1649)}$ are indicated. at 21°C with various molar excess of peptides L or K (Genemed Synthesis, Inc.) in incubation buffer (150 mM NaCl, 2 mM EGTA, 5 mM HEPES, pH 7.3) containing 50 nM ('low Ca²⁺' buffer) or 5 μ M [Ca²⁺] ('high Ca²⁺' buffer). Electrophoresis was carried out in 12% polyacrylamide gels in the same buffers under non-denaturing conditions. Gels were stained by Coomassie blue.

2.5. Fluorescence spectra

Fluorescence measurements were carried out on a luminescence spectrometer LS50B (Perkin Elmer Instruments). All spectra were corrected for background fluorescence.

3. Results

3.1. The C-terminal tail of the $\alpha_{1C} Ca^{2+}$ channel binds CaM in the presence and in the absence of Ca^{2+} ions

A GST pulldown assay using a fusion protein of GST and the C-terminal part of α_{1C} have shown (Fig. 1) that the α_{1C}



Fig. 2. Mutation of the $\alpha_{1C,77}$ channel to $\alpha_{1C,77L}$ impaired effects of Ca^{2+} and CaM on inactivation. A: Normalized Ba^{2+} and Ca^{2+} currents through $\alpha_{1C,77}$ and $\alpha_{1C,77L}$ co-expressed with auxiliary subunits and either CaM_{WT} or CaM_Q . Maximum currents were elicited by 300 ms depolarizations from a holding potential of -80 mV. B: Respective fractions of the currents (mean ± S.E.M. of three or four experiments) remaining at the end of 300 ms depolarization (r_{300}). Differences between r_{300} values of Ba^{2+} and Ca^{2+} currents are significant (asterisk, P < 0.01) only with $\alpha_{1C,77}$ channels co-expressed with CaM_{WT} . C: Voltage-dependence of Ba^{2+}/Ca^{2+} inward currents (\blacksquare) and their inactivation time constants (\blacktriangle) for $\alpha_{1C,77}$ channels co-expressed with CaM_{WT} or CaM_Q . A monotonic decrease in inactivation time constants with increasing voltage-dependent inactivation. An U-shape voltage-dependence of τ , observed only in Ca^{2+} -containing solution with $\alpha_{1C,77}$ channels to Ca^{2+} -dependent inactivation on Ba^{2+} currents through $\alpha_{1C,77}$ channels was better fit by one time constants with the fast ones shown. (mean ± S.E.M. of five or six experiments).

segment (1414–1649) incorporating motifs L and K, binds CaM not only in the presence of Ca²⁺ [13] but in some preparations also in Ca²⁺-free buffer (2 mM EGTA). Repeated experiments on GST- $\alpha_{1C(1414-1649)}$ suggest critical importance for the expressed protein of either correct folding or prior removal of bound Ca²⁺ to reveal CaM binding in Ca²⁺-free media. This finding suggests the presence of an additional Ca²⁺-independent tethering site for CaM in the C-terminal tail of the α_{1C} channel besides the Ca²⁺-dependent CaM-binding IQ region.

3.2. CaM is essential for Ca²⁺-induced inactivation mediated by motif L (1572–1587) of the C-terminal tail of α_{1C}

To establish whether motif L plays a role in CaM-modulation of Ca²⁺-induced inactivation, we compared the $\alpha_{1C.77}$ channel and its motif L-mutated isoform $\alpha_{1C,77L}$ [10] transiently co-expressed in tsA201 cells with wild-type CaM_{WT} or its inactive derivative CaMo [15] in which four Ca²⁺-binding EF-hand motifs were mutated. Whole-cell recordings of Ca²⁺ and Ba²⁺ currents (Fig. 2) showed characteristic acceleration of inactivation of the $\alpha_{1C,77}$ channel by CaM_{WT} but not CaM_Q, when Ca²⁺ was substituted for Ba²⁺ as charge carrier. Under the same conditions, the $\alpha_{1C,77L}$ channel containing an intact CaM-binding IQ region exhibited only a small Ca²⁺dependent increase in inactivation which was rather unrelated to the type of CaM co-expressed. Analysis of residual currents $(r_{300}, \text{ Fig. 2B})$ and voltage-dependence of inactivation time constants (τ , Fig. 2C) showed characteristics typical of Ca²⁺-induced inactivation for the $\alpha_{1C,77}$ channel co-expressed with CaM_{WT} but not CaM_Q. In contrast, neither a significant increase in r_{300} (Fig. 2B) with Ca²⁺ as charge carrier nor the classical U-shape of voltage-dependence of inactivation time constants (Fig. 2D) was found for the $\alpha_{1C,77L}$ channel co-expressed with either CaM_{WT} or CaM_Q. These data confirm our

earlier conclusion [10] of independent involvement of motifs L and K in Ca^{2+} -induced inactivation and suggest that these motifs may be differentially modulated by CaM.

3.3. CaM binds to the C-terminal region of motif L of the α_{1C} channel

To verify directly whether motif L interacts with CaM, we used synthetic peptides (Fig. 3A) which have been designed based on our segmental mutation analysis of Ca²⁺-induced inactivation of the α_{1C} channel [10]. LA peptide represents full amino acid sequence of the motif L, while shorter peptides LM1 and LM3 comprise N- and C-terminal parts of motif L (Fig. 3A) and, respectively, incorporate M1 and M3 segments critical for Ca²⁺-induced inactivation [10]. For comparison, we used peptide L⁸⁶ (RETELSSQVQYQAKEA), which contains amino acids replacing motif L in $\alpha_{1C,77L}$ [10]. Fig. 3B shows results of the PAGE shift assay under non-denaturing conditions of CaM preincubated with peptides in media containing physiologically relevant 'resting' (50 nM) and 'active' (5 μ M) [Ca²⁺]. In low Ca²⁺ medium (50 nM [Ca²⁺]), both peptides LA and LM3 bind to CaM thus increasing its size which caused a decrease in electrophoretic mobility of the complex on a non-denaturing PAGE (Fig. 3B, left). Since both LA and LM3 carry high positive charge at neutral pH (pI 10.2 and 9.9, respectively), they have tendency to move to cathode in upward direction out of the gel. Efficiency of CaM binding therefore can be estimated by a reduction of the CaM band with the increase in peptide/CaM molar ratio. Peptide LM1, however, carries net negative charge at neutral pH (pI=4.9) and its position on the gel (marked by an arrow) is clearly seen. However, CaM band remains equally stained by Coomassie blue even at very high LM1/CaM ratios (Fig. 3B, lower left). In high Ca²⁺ medium, both peptides LA and LM3 retain their CaM-binding property. Notable CaM gel



Fig. 3. CaM interacts with the C-terminal half of the motif L of the α_{1C} Ca²⁺ channel tail. A: Amino acid sequences of the motif L-derived peptides. M1 and M3 denote identified earlier [10] segments critical for Ca²⁺-induced inactivation of the channel. B: PAGE-shift assay demonstrating Ca²⁺-dependence of CaM interaction with peptides LA and LM3 in low [Ca²⁺] (left) and high [Ca²⁺] (right) media at different peptide:CaM ratios. Arrows show unbound CaM and LM1.



Fig. 4. Effect of divalent cations on motif L-derived peptides. A: Emission fluorescence spectra of peptide LM1^{fl} (250 nM in 2 mM EGTA, 100 mM NaCl and 10 mM HEPES, pH 7.38; $\lambda_{ex} = 494$ nm) demonstrating lack of the effect of 250 nM CaM after a 30 min incubation, and $\approx 50\%$ decrease of the peak fluorescence by 1 μ M [Ca²⁺]. B: Dependence of normalized peak fluorescence of peptide LM1^{fl} at 519 nm on [Ca²⁺] (\odot), [Ba²⁺] (\odot), [Mg²⁺] (\bigstar) or [Ca²⁺] in the presence of 1.8 mM [Mg²⁺] (\boxdot) (23°C). For comparison, dependence of normalized peak fluorescence of peptide LA^{fl} at 519 nm on [Ca²⁺] is shown (\Box). Curves were fitted by equation $y = 1 - (1/(1+(K_d/x)^n)))$, where *n*: Hill coefficient. [Cation²⁺] was calculated using on-line program Webmaxc2 (www.stanford.edu/~cpatton). C: Fluorescence spectra of Trp¹⁵⁹³ in LA in low Ca²⁺ medium (1), in the presence of 14 μ M CaM (2) and in the presence of 14 μ M CaM+1 μ M [Ca²⁺] (3). Shown are also spectra of CaM (2 μ M) in low Ca²⁺ medium (4) and in 1 μ M [Ca²⁺] (5); $\lambda_{ex} = 295$ nm. D: Fluorescence spectra of Trp¹⁵⁹³ in LM3 in low Ca²⁺ medium (1), in the presence of 14 μ M CaM+1 μ M [Ca²⁺] (3); $\lambda_{ex} = 295$ nm. All spectra are corrected for background fluorescence. E: Dependence of normalized peak Trp¹⁵⁹³ fluorescence of LA (\odot) and LM3 (\blacksquare) on [Ca²⁺] > 1 μ M, LM3 shows significant contribution of the lower affinity binding which was not immediately apparent in LA and is not further discussed.

shifts caused by LA in 5 μ M [Ca²⁺]_i (Fig. 3B, upper right) occur at lower molar ratio than in low Ca²⁺ medium (Fig. 3B, upper left) suggesting higher affinity of CaM to LA in 5 μ M [Ca²⁺]_i. In high Ca²⁺ medium, LM1 did not cause any visible gel shift of CaM (Fig. 3B, lower right). Under none of these conditions, did the control peptide L⁸⁶ exhibit appreciable binding to CaM (not shown). These data are in accordance with the GST pulldown experiment (Fig. 1) and suggest that

the C-terminal portion of motif L may serve as a tethering site for CaM at both resting (closed) and active (open) states of the channel when $[Ca^{2+}]_i$ rise from low (50–100 nM) to high (1–5 μ M) levels.

3.4. Ca^{2+} sensor in the N-terminal region of motif L of the α_{1C} channel

Interestingly, free peptide LM1 did not enter the gel in 5 µM

[Ca²⁺] (Fig. 3B, lower right). This could be due to an interaction with Ca2+, which would change the peptide's net charge (-1 at pH 7) to positive at neutral pH. To investigate whether motif L is indeed sensitive to Ca^{2+} , we have studied the Ca²⁺-dependence of fluorescence of the N-terminally fluorescein-labeled peptides LM1^{fl} and LA^{fl} (Fig. 4). The peak fluorescence of LM1^{fl} was quenched by Ca²⁺ independently of CaM (Fig. 4A) with apparent K_d of 71 ± 4 nM and a Hill coefficient of 1.3 ± 0.1 (*n*=4) (Fig. 4B, \bigcirc). Physiological 1.8 mM $[Mg^{2+}]$ slightly shifted the $[Ca^{2+}]$ -dependence to $K_d = 101 \pm 2$ nM (n = 3; Fig. 4B, \blacksquare), i.e. to the range typical for the resting $[Ca^{2+}]_i$. Much weaker specificity for Ba^{2+} $(K_d \approx 1,7 \text{ mM})$ or Mg^{2+} $(K_d \approx 93 \text{ mM})$ determined from the peptide LM1^{fl} titration at 519 nm (Fig. 4B) conforms to the lack of these ions effect on Ca^{2+} channel inactivation. Essentially similar data were obtained for LAfl, which fluorescence was quenched by Ca2+ independently of CaM with apparent K_d of 147 ± 3 nM (Fig. 4B, \Box). These data suggest that motif LM1 is a new, highly selective Ca²⁺ sensor of the channel.

3.5. Possible role of Ca^{2+} sensor for CaM binding and Ca^{2+} -induced conformational change of motif L of the α_{1C} channel

Presence of a single Trp¹⁵⁹³ residue in the C-terminal CaMbinding region of peptide LA allows to monitor conformational rearrangements in the vicinity of this amino acid that would change its fluorescence properties. Maximum of fluorescence of Trp¹⁵⁹³ in peptide LA was observed at \approx 380 nm (Fig. 4C, curve 1), which suggests exposure of this chromophore to hydrogen bonding groups or water. Since peptide LM3 lacking the 11 amino acid N-terminal sequence of LA (Fig. 3A) has the same maximum as peptide LA (Fig. 4D, curve 1), Ca²⁺ sensor does not appear to contribute to the spectral properties of the Trp¹⁵⁹³. Fluorescence spectra of neither LA nor LM3 were appreciably changed in the presence of 2 mM [Ca²⁺] thus indicating an apparent lack of a conformational link between the N-terminal Ca²⁺ sensor and C-terminal CaM-binding region of LA.

Titration of LA with CaM, which does not contain Trp residues, in low $[Ca^{2+}]$ medium caused blue shift of the spectral maximum of Trp^{1593} that may reflect its shielding due to the binding of CaM. Indeed, changes in the peak fluorescence of Trp^{1593} induced in LA (Fig. 4C, curve 2) and LM3 (Fig. 4D, curve 2) by CaM in 50 nM [Ca²⁺] occur with very similar apparent K_d of 3.6 ± 0.2 and $3.0 \pm 0.3 \mu M$, respectively. To find whether the presence of Ca2+ sensor in the N-terminal part of LA does affect Ca²⁺ sensitivity of the complex of motif L with CaM, we monitored changes in Trp¹⁵⁹³ fluorescence in LA and LM3 in the presence of CaM upon titration with Ca²⁺ (Fig. 4C,D, curves 3). In both peptides, Ca2+ induced increase and shift of the maximum of Trp¹⁵⁹³ fluorescence to 328 nm, which occurred with apparent K_d of 145±3 (peptide LA, \bullet) and 282±10 nM (peptide LM3, ■) (Fig. 4E). Thus, Ca²⁺ sensor determines higher Ca²⁺ sensitivity of the interaction of CaM with motif L. This result suggests that CaM may help to extend Ca^{2+} induced conformational changes in Ca²⁺ sensor downstream the α_{1C} channel tail to the CaM binding site, which may have direct impact on Ca²⁺-dependent inactivation of the channel.

4. Discussion

It appears that CaM, probably only in its Ca²⁺-bound form, is able to transduce the conformational changes caused in Ca^{2+} sensor by the permeating Ca^{2+} ions, when $[Ca^{2+}]$ rises above the resting ≈ 100 nM. An immediately apparent result of this CaM-mediated transduction is a two-fold increase in the affinity of the CaM binding site of motif L monitored as a dramatic change in surroundings of Trp¹⁵⁹³. The combined effect of Ca²⁺ sensor and CaM may be of critical importance for the Ca²⁺-induced inactivation of the α_{1C} channel. Indeed, segment-exchange mutation in either Ca²⁺ sensor or CaMbinding regions of motif L each caused only partial loss of Ca²⁺-induced inactivation [10] indicating that neither of the two structures alone is ultimately critical for the Ca²⁺-dependence of the current decay. Only when applied together, did these mutations produce complete loss of Ca²⁺ sensitivity and its CaM-dependence as it is shown in Fig. 2 for the $\alpha_{1C,77L}$ channel.

The combined effect of Ca^{2+} on Ca^{2+} sensor of motif L in the resting cell ($K_d \approx 100 \text{ nM}$) together with a large conformational change of CaM [17-19] from the extended shape to a globular structure wrapping around the target peptide (upon binding of 3 or 4 Ca^{2+} when $[Ca^{2+}]_i$ increases above 500 nM) provides further insight into the complex nature of Ca²⁺-dependent inactivation. Given that the L region is conserved among all dihydropyridine-sensitive Ca²⁺ channels of class C, D and S, the two-site modulation by Ca²⁺ and CaM that we propose here may be general to their Ca²⁺-dependent regulation. The question of how the rise in $[Ca^{2+}]_i$ is transduced by CaM into inactivation of the channel may require major crystallographic and NMR studies to be elucidated. We have shown recently that mutations in the CaM-binding L or K regions leading to removal of Ca²⁺-induced inactivation reduce single channel conductance by 15-20% [20]. These data may reflect interaction between the pore region and cytoplasmic L/K sequences of the channel. A disruption of this interaction leading to the closing of the channel may be promoted by an involvement of the recently described [21] VVTL sequence in the EF-hand region, or to other yet unidentified positions that may be closer to the pore.

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