C-terminal Fragments of the α₁C (Caᵥ1.2) Subunit Associate with and Regulate L-type Calcium Channels Containing C-terminal-truncated α₁C Subunits*

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L-type Ca²⁺ channels in native tissues have been found to contain a pore-forming α₁ subunit that is often truncated at the C terminus. However, the C terminus contains many important domains that regulate channel function. To test the hypothesis that C-terminal fragments may associate with and regulate C-terminal-truncated α₁C (Caᵥ1.2) subunits, we performed electrophysiological and biochemical experiments. In tsA201 cells expressing either wild type or C-terminal-truncated α₁C subunits in combination with a β₃a subunit, truncation of the α₁C subunit by as little as 147 amino acids led to a 10–15-fold increase in currents compared with those obtained from control, full-length α₁C subunits. Dialysis of cells expressing the truncated α₁C subunits with C-terminal fragments applied through the patch pipette reconstituted the inhibition of the channels seen with full-length α₁C subunits. In addition, C-terminal deletion mutants containing a tethered C terminus also exhibited the C-terminal-induced inhibition. Immunoprecipitation assays demonstrated the association of the C-terminal fragments with truncated α₁C subunits. In addition, glutathione S-transferase pull-down assays demonstrated that the C-terminal inhibitory fragment could associate with at least two domains within the C terminus. The results support the hypothesis that the C-terminal fragments of the α₁C subunit can associate with C-terminal-truncated α₁C subunits and inhibit the currents through L-type Ca²⁺ channels.

The voltage-activated L-type Ca²⁺ channels are heteromeric proteins minimally composed of a pore-forming α₁ subunit and accessory α₂δ and β subunits (1, 2). Each α₁ subunit contains four repeated domains containing a total of 24 membrane-spanning domains as well as a long hydrophilic C terminus which contains important regulatory domains that contribute to channel regulation. For example, the C terminus of the α₁C subunit constitutes ~30% of the total mass of the α₁C subunit (3) and is critical for membrane targeting of the channels (4), the regulation of the channels by protein phosphorylation (5), and the binding of Ca²⁺-binding proteins such as calmodulin and sorcin (6–8). In addition, the C terminus of α₁C appears to contain inhibitory domains because deletion of up to ~70% of the C-terminal 665 amino acids leads to increased currents (9).

A puzzling observation that has been made in several laboratories is that the C terminus of several L-type Ca²⁺ channels appears to be truncated in many native tissues. For example, when the α₁C subunit was isolated from cardiac myocytes, only 10–15% of the total protein was a full-length 240-kDa α₁C subunit, whereas the majority migrated on SDS gels as a ~190-kDa protein that was lacking the distal ~50 kDa of the C terminus (10). Similar observations have been made for the α₁C subunit expressed in brain (11) and the α₁S subunit isolated in vitro into a “body” of 190 kDa, similar to what is observed in native systems (10), and C-terminal fragments of 30–50 kDa that remained associated with the membrane (17). Here we report studies in which we have tested the ability of C-terminal fragments to associate with and regulate the conductance of C-termnially cleaved α₁C subunits expressed in intact cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents were obtained from general sources unless otherwise stated. Antibodies Card I, Card C (15), and CT11,2 as well as protein G-agarose (50 ml, 5 mg/ml) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The γ-[³⁵S]ATP generator and [⁴³Ca]Cl₂ were purchased through the Midwest Radioisotope Services, Inc. (Chicago, IL). Myoglobin was purchased through Sigma. hen egg lysozyme was purchased through Calbiochem-Novabiochem (San Diego, CA). Anti-FLAG M2 antibody was purchased from Sigma. Anti-GST antibody was purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). In vitro translation reagents were purchased from Promega (Madison, WI). The Diacyl-GMP assay kit was obtained through Sigma. The C-terminal fragments of the α₁C subunit used in this study were purified as described previously (6). The C-terminal fragments of the α₁S subunit were purified as described previously (15).

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‡ The abbreviations used are: CT, C-terminal domain; GST, glutathione S-transferase.
the expression vectors pCR3αC, pCR3αCΔ2024, pCR3αCΔ1905, pCR3αCΔ1733, pCR3αCΔ1623, pCR3αCΔ1733–1905, and pCR3αC (4) were described previously. The vectors pCR3αCΔ1733–2024 and pCR3αCΔ1905Δ2024 were prepared using strategies similar to those described earlier (4). A schematic diagram depicting the various constructs is shown in Figure 1.

Antibody Preparation—To generate an additional antibody that would recognize C-terminal fragments of the αC subunit, a fusion protein encoding amino acid residues 1907–2171 (termed CT4, see Fig. 1B) in the C terminus of the αC subunit was produced. The sequence in the CT4 region was subcloned into an expression vector pQE32 (Qiagen) as described (15, 20). For electro- and either the calcium phosphate precipitation method or the transfection reagent Effectene from Qiagen as described (15, 20). For electro- and either the calcium phosphate precipitation method or the transfection reagent Effectene from Qiagen as described (15, 20). For electro-

To express C-terminal fragments of CT4, CT7, CT8, CT12, CT14, CT23 (see Fig. 1C), wild-type constructs used in this study is shown in Fig. 1.

Expression of Channel Subunits and C-terminal Fragments of the αC Subunit in Mammalian Cells—Various C-terminal fragments of the αC subunit (see Fig. 1) were expressed in tsA201 cells (HEK293 cells transformed with large T-antigen (18)) using the following strategies. The C-terminal domain (CT) containing amino acids 1623–2171 of αC was excised as a BglII/BamHI fragment. The CT (containing amino acid residues 1623–2171) was subcloned into pCR3αC. Fragments were derived from pCR3αC and pCR3αCΔ1905 constructs as a BamHI/BamHI and BglII/BamHI fragments, respectively. The CT fragment containing amino acids 2024–2171 was derived from pCR3αCΔ2024 construct as a BamHI/BamHI fragment. A fusion protein expression vector, pCR3×His/Myc, was derived from pCR3 (Invitrogen) by inserting a 6×-His tag and six copies of the Myc epitope into the multiclional sites of the original vector to allow expression of a protein with both 6×-His- and Myc tag fused to the N terminus. To create expression constructs encoding different regions of the C terminus of the αC subunit, the cDNA fragments of CT, CTD, and CT23 were subcloned into the BamHI-digested pCR3His/Myc vector, resulting in in-frame fusions of the C-terminal fragments to the 6×-His and six copies of Myc tags on the vector. TsA201 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc. containing 10% fetal bovine serum (Life Technologies, Inc. and 1% penicillin/streptomycin at 37 °C in 5% CO2. Transient expression of the Myc-tagged C-terminal fusion proteins in tsA201 cells along with wild type or mutant rabbit αC (Ca.1,2) subunits (3) and the rat βα C subunit (19) was carried out using a total of 30–40 μg of plasmid DNA/100-mm plate and 400 μg of calcium phosphate precipitation. The transfection reagent Effectene from Qiagen was described (15, 20). For electrophysiological experiments, tsA201 cells were plated onto 6-cm culture dishes to achieve ~40–60% confluency. The indicated αC subunit construct cDNAs were co-transfected with βα C subunit cDNA at 1.5 μg each along with 0.2 μg of CD8 cDNA (as an expression indicator (21)). On the day of the experiment transfected cells were washed with phosphate-buffered saline, dissociated using trypsin-EDTA (Life Technologies, Inc.) and transferred onto either 35-mm culture dishes or 12-mm glass coverslips, each previously coated with rat tail collagen

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Electrophysiological Assays—Ba2+ currents were obtained at room temperature (~22 ± 1 °C) from the transfected human embryonic kidney cells using the whole cell patch voltage clamp technique. The currents were recorded utilizing an EPC-7 patch clamp amplifier (List) whose analog output signal was low pass-filtered at 3 kHz and then digitally sampled at 5 kHz using an IS-2 data acquisition system and Digitizer (Datalab, Frankfurt/Main, Germany). Data were acquired on a Keithley 1905 subunit that lacks the CT4 fragment (Fig. 1), and CT4 expressed in tsA201 cells (see Fig. 1C).
RESULTS AND DISCUSSION

Progressive C-terminal Deletions of the Full-length α1C L-type Ca\(^{2+}\) Channel Results in Potentiated Channel Currents—

Previous studies demonstrate that truncation of 307–472 amino acids from the C terminus of α1C (in mutants α1CΔ1856, α1CΔ1733, and α1CΔ1700) produced increased channel currents in Xenopus oocytes (9), suggesting that the C terminus contains inhibitory elements. To test the hypothesis that C-terminal fragments of the α1C subunit might associate with and regulate the conductance of C-terminal-truncated α1C subunits, we determined if expressed C-terminal fragments could reconstitute the inhibition of currents when co-expressed with C-terminal-truncated mutants of α1C. To do so, we first prepared and analyzed currents from several different mutants. Using 10 mM Ba\(^{2+}\) as a charge carrier, whole cell current density was compared from channels containing the full-length α1C subunit or from the C-terminal deletion mutants α1CΔ2024, α1CΔ1905, and α1CΔ1733 (see Fig. 1). Each construct was transiently expressed in tsA-201 cells, and all channel constructs were co-expressed with the rat β2 subunit (19). The current-voltage relationships of channels containing full-length α1C, α1CΔ2024, α1CΔ1905, or α1CΔ1733 were determined (Fig. 2). The full-length channel displayed a characteristic L-type current-voltage profile with the maximal peak I\(_{\text{Ba}}\) at 0 mV and a mean current density of 4.97 ± 1.3 (mean ± S.E., n = 4) pA/pF. In marked contrast, all three truncation mutants displayed significantly larger currents at all voltages between −20 and +40 mV. The current-voltage (I-V) relationship obtained from cells expressing the α1CΔ2024 subunit displayed maximal current at +10 mV with an average peak I\(_{\text{Ba}}\) of 60.1 ± 19.2 pA/pF (n = 5). Similarly, currents from cells expressing the α1CΔ1905 or α1CΔ1733 subunits also exhibited peak currents between 0 to +10 mV with current densities of 59.3 ± 12.9 pA/pF (n = 4) and 44.7 ± 13.4 pA/pF (n = 6), respectively. These results demonstrated that expression of any of the three truncation mutants gave rise to markedly larger currents in mammalian cells in a manner similar to that previously described for the truncation mutants α1CΔ1856, α1CΔ1733, and α1CΔ1700 that were expressed in Xenopus oocytes. More importantly, the results demonstrated that a C-terminal truncation of the α1C subunit by as little as 147 amino acids (α1CΔ2024) displayed markedly enhanced currents when compared with the intact full-length channel. This suggested that the inhibitory motif might be contained between amino acids 2025 and 2171.

Cytosolic Application of α1C C-terminal Fragments to Cells Expressing Truncated α1C Subunits Resulted in Time-dependent Reduction of I\(_{\text{Ba}}\)—We next tested if application of C-terminal fragments would inhibit the C-terminal-truncated α1C subunits. We first tested if the CT fragment, corresponding to amino acids 1507, or the C-terminal deletion mutants α1CΔ2024 and α1CΔ1905. GST-CT was applied through the patch clamp pipette at a peptide concentration of 1 μg/μl pipette solution. During recordings, cells were maintained at −90 mV holding potential and depolarized to +10 mV for 50 ms at 10-s intervals. In the control cells, currents in both the α1CΔ1905 (Fig. 3) and α1CΔ2024 (data not shown) expressing cells exhibited a time-dependent increase in currents that began immediately upon patching the cells. Peak currents were achieved within ~3 min after access to the cell was achieved and were maintained with minimal decrease (~10%) during the 15-min recording period (Fig. 3). In cells exposed to CT peptide currents also showed an initial increase in current density (Fig. 4). However, a reduced peak, compared with control, was attained within 2 min of open access to the cytosol (Fig. 4). Current continued to decline such that I\(_{\text{Ba}}\) was markedly reduced compared with control after a 6–10 min exposure of either α1CΔ2024 or α1CΔ1905 to CT (Fig. 4, A and B, and Table I). The inhibitory effect of CT was essentially abolished when the CT peptide was boiled for 5 min before addition to pipette solution (Table I). These data demonstrated that the inhibition of channels could be reconstituted by applying the CT peptide to cells expressing the C-terminal-truncated mutants, α1CΔ2024 and α1CΔ1905. Furthermore the structural integrity of the peptide was necessary for the peptide to exert its inhibitory effect. These data provided initial support for the hypothesis that C-terminal fragments of α1C can associate with and regulate channel activity in intact cells.

The Distal Portion of the C-terminal Tail Contains a Channel
Inhibitory Domain—We next attempted to define smaller regions of the C terminus that might be responsible for channel inhibition. We focused initially on fragments CT4 and CT7, which were approximately equivalent to the fragments deleted from α1CΔ1905 and α1CΔ2024, respectively (see Fig. 1). Application of the GST-CT4 peptide to cells expressing either α1CΔ2024 or α1CΔ1905 caused a marked inhibition of currents in a manner similar to the CT peptide (Fig. 5 and Table I). An early and reduced current peak was observed within 4 min of application of CT4; current density was markedly reduced compared with control in α1CΔ2024- and α1CΔ1905-expressing cells from 4 to 10 min after the start of the perfusion (Fig. 5, A and B, Table I). Boiling the CT4 peptide again abolished the inhibitory effect (Table I).

Similar experiments were performed with GST-CT7. Surprisingly, GST-CT7 was without effect on currents from cells expressing either α1CΔ2024 or α1CΔ1905. However, since truncation of the CT7 fragment in the α1CΔ2024 mutant resulted in a loss of inhibition (Fig. 2), we next asked if a different type of CT7 fusion protein, 6×-His-CT7, could inhibit the C-terminal-truncated mutants. Conceivably, the GST construct may have hampered the presentation of CT7 to the channels. Application of 6×-His-CT7 to cells expressing α1CΔ1905 caused a marked reduction from control within 6–8 min after access to the cytosol (Fig. 6A, Table I). The 6×-His-CT7 also caused inhibition of currents from α1CΔ2024 (Fig. 6B, Table I). The effects of CT7 were similar to those of CT and CT4, although the inhibition appeared to be less robust than that caused by CT4. In particular, the inhibition of currents from α1CΔ2024 by CT7 appeared to develop more slowly and to a smaller extent that that caused by CT4 (compare Figs. 5 and 6, Table I). Nevertheless, these results demonstrated that application of CT7 could reconstitute inhibition of currents from either α1CΔ1905 or α1CΔ2024. The effects of CT7 were resistant to boiling. It is well known that the activity of many small proteins, such as calmodulin, can be resistant to denaturation (24), and thus, the lack of inhibition of the Ba2+ current during intracellular dialysis with the small, boiled 6×-His-CT7 was not surprising.

We also tested the effects of other peptides derived from the C terminus of α1C. As a control, we tested GST alone and found it had no effects on the currents (Fig. 7, A and B, Table I). Peptide GST-CT23, corresponding to amino acids 1622–1905, had no effects on the currents (Fig. 7, A and B, Table I). Since CT4 appeared to be more efficacious than CT7 (the C-terminal half of CT4), we tested whether CT8, which corresponded to the N-terminal half of CT4 (amino acids 1905–2024, Fig. 1), had any inhibitory activity. Conceivably CT4 might be a better inhibitor because an additional inhibitory domain might be contained within the CT8 fragment. However, neither GST-CT8 (data not shown) nor 6×-His-CT8 (Fig. 7, A and B, Table I) had any effect on channel currents. Taken together, these results indicated that the inhibitory effect of the C terminus could be reconstituted by applying fragments as small as CT7 (corresponding to the most distal 144 amino acids of the C terminus) to cells expressing truncated α1C subunits.

The more effective inhibition of currents by CT4 compared with CT7 may have been due to a more effective association of CT4 with the truncated channels (see below).
The effects of C-terminal fragments on peak \( I_{\text{Ba}} \) density are shown. Control groups were performed in parallel with experimental conditions, and indicated values are presented for the appropriate series of experiments. For each construct average current values (normalized to cell capacitance) are displayed for the indicated condition as well as the ratio values of \( I_{\text{Ba}} \) of 8 min and 2 min (r8:2') for each individual recording. The values marked with an footnote symbol were significantly different from control.

### Table I

| Construct | \( \alpha_{1C}\Delta 1905 \) | \( \alpha_{1C}\Delta 2024 \) |
|-----------|-------------------------------|-------------------------------|
|           | \( I_{\text{Ba}} \) (mean ± S.E.) | \( (r8:2') \) | \( I_{\text{Ba}} \) (mean ± S.E.) | \( (r8:2') \) |
| Control   | -44.4 ± 3.60                  | 1.13 ± 0.08                  | -57.6 ± 4.03                  | 0.92 ± 0.04                  |
| CT        | -25.5 ± 4.84*                 | 0.63 ± 0.13                  | -20.1 ± 2.57*                 | 0.46 ± 0.01                  |
| CT boiled | -48.4 ± 1.93                  | 0.95 ± 0.01                  | -48.9 ± 1.88                  | 0.88 ± 0.06                  |
| Control   | -57.8 ± 7.39                  | 1.05 ± 0.06                  | -49.9 ± 3.81                  | 0.98 ± 0.08                  |
| CT4       | -13.1 ± 6.98*                 | 0.23 ± 0.14                  | -15.0 ± 5.13*                 | 0.26 ± 0.11                  |
| CT7       | -22.1 ± 9.78*                 | 0.32 ± 0.17                  | -35.2 ± 6.15*                 | 0.62 ± 0.10                  |
| CT23      | -49.8 ± 2.88                  | 0.98 ± 0.07                  | -53.5 ± 4.24                  | 1.08 ± 0.03                  |
| GST       | -46.2 ± 5.12                  | 0.92 ± 0.05                  | -52.6 ± 3.72                  | 0.98 ± 0.08                  |
| CT7 boiled| -29.5 ± 2.11                  | 0.70 ± 0.05                  | -23.7 ± 2.99                  | 0.62 ± 0.03                  |
| Control   | -45.8 ± 7.97                  | 0.98 ± 0.03                  | -39.9 ± 3.05                  | 0.90 ± 0.06                  |
| CT4 boiled| -41.0 ± 2.20                  | 0.91 ± 0.01                  | -42.5 ± 3.05                  | 0.97 ± 0.08                  |
| Control   | -59.2 ± 9.42                  | 1.02 ± 0.03                  | -54.3 ± 2.56                  | 0.92 ± 0.09                  |
| CT8       | -55.6 ± 4.12                  | 1.09 ± 0.06                  | -57.2 ± 4.32                  | 1.01 ± 0.07                  |

* \( p < 0.01. \)  
  ** \( p < 0.05. \)

**Fig. 5.** Inhibition of \( I_{\text{Ba}} \) currents by application of CT4. Time course of peak currents from cells expressing either the \( \alpha_{1C}\Delta 1905 + \beta_{2a} \) (A) or \( \alpha_{1C}\Delta 2024 + \beta_{2a} \) subunits (B) in the presence or absence of GST-CT4 (1 \( \mu \) g peptide/ml pipette solution) that was dialyzed into the cell via the patch clamp pipette. Results shown are the means ± S.E., with the number of experiments shown in parentheses.

**Fig. 6.** Inhibition of \( I_{\text{Ba}} \) currents by application of CT7. Conditions were as in Fig. 5 except that 6×His-CT7 (1 \( \mu \) g peptide/ml pipette solution) was added through the pipette. Results shown are the means ± S.E., with the number of experiments shown in parentheses.

CT7, both at the concentration (1 \( \mu \) g/ml, data not shown) that caused inhibition of \( \alpha_{1C}\Delta 2024 \) and \( \alpha_{1C}\Delta 1905 \) and at a 4-fold higher concentration (Fig. 8, Table I) caused little or no inhibition of currents from \( \alpha_{1C}\Delta 1905 \). These results suggested that the section of the C terminus between amino acids 1733 and 1905 was necessary for the inhibition caused by pipette application of either CT4 or CT7. Conceivably amino acids 1733–1905 might be the “receptor” for CT7 that allows for channel inhibition. Alternatively, CT7 might interact with another domain to cause inhibition, and amino acids 1733–1905 might play a role in helping to present CT7 to the inhibitory receptor.

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*Inhibitory Peptides*—The CT7 and CT4 peptides induced functional inhibition of currents generated through \( \alpha_{1C}\Delta 2024 \) and \( \alpha_{1C}\Delta 1905 \). We asked whether the inhibition could be produced with an \( \alpha_{1C} \) subunit that was truncated farther upstream, such as the \( \alpha_{1C}\Delta 1733 \) truncation mutant. Interestingly, application of CT4 to cells expressing the \( \alpha_{1C}\Delta 1733 \) truncation mutant caused a modest inhibition, but this was not significantly different from control (Fig. 8, Table I). Similarly,
As such, amino acids 1733–1905 might act to anchor or stabilize the inhibitory domain within CT7.

To test the above possibilities, we determined whether constructs containing a “tethered” CT4 or CT7 domain might exhibit the inhibited currents. Two additional constructs were made that contained internal deletions within the C terminus of the fragment corresponding to amino acids 1733–1905 and 2024–2171 (α1CΔ1733–1905Δ2024) (see Fig. 1). Current-voltage relationships were determined as described in the legend to Fig. 2. Results are the means ± S.E. with the number of experiments shown in parentheses. Neither CT4 nor CT7 produced statistically significant effects. Ctrl, control.

TABLE II

| Construct | Peak currents (x ± S.E.) |
|-----------|-------------------------|
| α1CΔ1733 | 46.5 ± 5.2              |
| α1CΔ1733–1905 | 5.4 ± 1.1             |
| α1CΔ1733–2024 | 4.9 ± 0.6             |
| α1CΔ1733–1905Δ2024 | 52.5 ± 13.0          |

Data are peak currents from the experiments depicted in Fig. 9.
constructs lacking amino acids 1733–1905 suggested that CT7 interacted with something other than these amino acids to cause inhibition of the channels. However, since CT7 could not inhibit $\alpha_{1C}\Delta 1733$, which lacks these amino acids, the data are consistent with the idea that amino acids 1733–1905 play an important role in presenting CT7 to the inhibitory receptor. This concept suggests that CT7 might have multiple binding sites.

Co-immunoprecipitation of the C-terminal Fusion Proteins with the Channel Subunits—We performed biochemical studies to determine that CT4 and CT7 could associate with $\alpha_{1C}$ subunits in tsA cells. First, CT4 and CT7 in the pCR3His/Myc vector were transiently transfected into tsA201 cells, and expression was ascertained by SDS-polyacrylamide gel electrophoresis and immunoblotting. Staining with the anti-Myc or CT4 antibodies revealed expression of CT4 and CT7 (see Figs. 10 and 11). Next, we tested whether the C-terminal segments of the $\alpha_{1C}$ subunit could directly associate with the channel subunits. C-terminal fragments were co-expressed with full-length wild type $\alpha_{1C}$ or $\alpha_{1C}\Delta 2024$, $\alpha_{1C}\Delta 1905$, $\alpha_{1C}\Delta 1733$ subunits and the $\beta_{2a}$ subunits. Whole cell lysates were prepared from the transfected cells and immunoprecipitated with the CT2 antibody. When the channel subunits were co-expressed with CT4 and immunoprecipitated with the CI2 antibody, which is directed against the II-III loop of $\alpha_{1C}$ and does not recognize CT4, CT4 was co-immunoprecipitated with the $\alpha_{1C}$ subunits (Fig. 10). The $\alpha_{1C}$ subunits in the immunoprecipitates were detected on the blot using the Card I antibody (Fig. 10, upper panel), and the co-immunoprecipitated CT4 was detected by the anti-myc antibody (Fig. 10, lower panel). As a negative control, the CI2 antibody did not immunoprecipitate the CT4 fusion protein in the absence of the $\alpha_{1C}$ subunits (Fig. 10). Surprisingly, CT4 co-immunoprecipitated not only with $\alpha_{1C}\Delta 2024$ and $\alpha_{1C}\Delta 1905$ but also with $\alpha_{1C}\Delta 1733$ (Fig. 10), which was not inhibited by CT4 (Fig. 8). However, more CT4 was present in the immunoprecipitates with wild type $\alpha_{1C}$ and $\alpha_{1C}\Delta 2024$ or $\alpha_{1C}\Delta 1905$ compared with those containing $\alpha_{1C}\Delta 1733$. These results suggested that there might be multiple interaction sites for CT4 and that $\alpha_{1C}\Delta 1733$ lacks an interaction site that is necessary for channel inhibition. The results are consistent with the concept that amino acids 1733–1905 may be critical for allowing inhibition by CT4. The observation that CT4 could bind to full-length $\alpha_{1C}$ suggested that the interaction sites for CT4 were available in this protein.

In other experiments CT7 was co-expressed with either wild type $\alpha_{1C}\beta_{2a}$ or with the various C-terminal deletion mutants of $\alpha_{1C}$ in combination with $\beta_{2a}$ subunits in tsA cells. In contrast to CT4, the CT7 fusion protein did not co-immunoprecipitate with either the wild type or the mutant $\alpha_{1C}$ subunits (data not shown). However, because CT7 was effective in inhibiting channel activity but appeared less robust in causing inhibition than CT4 (Figs. 4 and 5; Table I), we reasoned that the association of CT7 might have been weaker and disrupted during the detergent solubilization and repeated washings of the immunoprecipitates. Thus, we asked if inclusion of a cross-linking agent during the immunoprecipitation might allow for detection of the association of CT7 with the channels. For these studies we used the cross-linking agent Ni(II)-GGH complex of the tripeptide NH2-Gly-Gly-His-COOH, which has been shown to be highly specific in that only proteins that specifically associated could be cross-linked (29). The lysates from cells expressing CT7 and mutant or wild type $\alpha_{1C}$ subunits were incubated with the cross-linking agent for 10 min at room temperature, and the reactions were quenched with thiourea. When the channels were immunoprecipitated with the CI2 (data not shown) or Card I antibodies (Fig. 11), both of which are directed against the internal II-III linker of $\alpha_{1C}$, and do not recognize CT7, CT7 was co-immunoprecipitated (Fig. 11). Taken together, these results demonstrated that the CT7 could directly associate with the channels in intact cells. In addition, the results supported the observations that, although the inhibi-

**Fig. 10.** Co-immunoprecipitation of CT4 with the channel subunits. TsA201 cells were co-transfected with CT4, the rat $\beta_{2a}$ subunit, and wild type or mutant $\alpha_{1C}$ subunits as indicated. Whole cell lysates were prepared from the transfected cells, and the channel subunits were immunoprecipitated with the CI2 antibody. For cells expressing CT4 alone, CT4 was concentrated by absorption onto a nickel resin. The immunoprecipitates (lanes 1–4) or concentrated lysates (lane 5) were electrophoresed on a 5–15% gradient (acylamide) SDS gel and transferred to a filter for immunoblotting. The filter was cut in half, and the immunoprecipitated $\alpha_{1C}$ subunits (wild-type or deletion mutants) were detected on the top portion of the immunoblot with the Card I antibody, whereas the co-immunoprecipitated CT4 fusion proteins were detected on the bottom portion using the anti-Myc antibody. The lane marked CT4 alone represents the concentrated CT4 from cells transfected with only this vector.

**Fig. 11.** Co-immunoprecipitation of CT7 with the channel subunits. TsA201 cells were co-transfected with CT7, the rat $\beta_{2a}$ subunit, and wild type or mutant $\alpha_{1C}$ subunits as indicated. Whole cell lysates were prepared from the transfected cells and subjected to cross-linking with the Ni(II)-GGH complex as described under “Experimental Procedures.” The lysates were diluted, and the channel subunits were immunoprecipitated with the Card I antibody. For cells expressing CT7 alone, CT7 was concentrated by absorption onto a nickel resin. Other conditions were as in Fig. 10. The Western blot was cut in half, and the immunoprecipitated $\alpha_{1C}$ subunits (wild-type or deletion mutants) were detected on the top portion of the immunoblot with the CI2 antibody, whereas the co-immunoprecipitated CT7 fusion proteins were detected on the bottom portion using the CT4 antibody. The lane marked CT7 alone represents the concentrated CT7 from cells transfected with only this vector.
147 amino acids, as in the a-terminal fragments. GST pull-down assays were performed as described under "Experimental Procedures" by applying purified 6×-His-CT7 to glutathione-Sepharose beads containing immobilized GST-CT (lane 1), GST-CT4 (lane 2), GST-CTB (lane 3), GST-CT12 (lane 4), GST-CT14 (lane 5), GST-CT23 (lane 6), GST-NT (lane 7), GST alone (lane 8), GST-L1 (lane 9), or GST-L2 (lane 10). The reactions were electrophoresed on a 15% acrylamide SDS gel and transferred to a filter for immunoblotting. The blot was probed for the presence of bound CT7 with the CT4 antibody.

Interactions of CT7 with the C Terminus of α1C in GST Pull-Down Assays—Since CT7 appeared to contain the inhibitory domain, it was of interest to identify the binding sites for CT7. GST pull-down assays were performed with bacterially expressed 6×-His-tagged CT7 and GST constructs derived from the intracellular domain of α1C. CT7 bound to GST constructs of CT, CT4, CT12, CT23, and CT14 but not to CT8, NT, L1, L2, or GST alone (Fig. 12). A common site shared by CT, CT4, CT12, and CT23 is contained within amino acids 1733–1905. These data along with those obtained from the electrophysiological and co-immunoprecipitation data suggest that one interaction site for CT7 is amino acids 1733–1905. However, CT7 also bound to CT14 (amino acids 1623–1733), which is upstream of 1733–1905. This is consistent with the idea that there are multiple interaction sites for CT7. That the CT14 site is upstream of amino acid 1733 is consistent with the result that CT7 associates with the inhibitory domain. It appears that the C terminus of the α1C subunit contains several regulatory domains within the C terminus that are identified and located schematically. Our previous work indicates that the C terminus of the majority of the full-length channel is cleaved proximal to the 1900-amino acid region into both proximal and distal portions. The present data (obtained using the CT4 and CT7 peptides) suggest that the portion of the distal C terminus between amino acid 2024 and 2171 (corresponding to CT7) serves as an inhibitory domain. The efficacy for inhibition is enhanced by the peptide region 1909–2024 (included in CT4 but absent from CT7). Inhibition mediated by the cleaved peptide inhibitory domain requires the peptide region between 1733 and 1905 on the proximal portion of the C terminus, and therefore, we suggest that this region contains a distal C terminus binding region. In this way the distal C terminus functions to inhibit and thus regulate channel conductance through and in response to an as yet undefined mechanism. Also, shown for completeness are several other known regulatory domains including the EF-hand, calmodulin binding domain (CBD) (7), the proline-rich domain (PRD) (17), and the Ser-1928 residue (the only site capable of in vivo phosphorylation) (5).

Many studies demonstrate that the α1C subunit appears to be truncated at the C terminus to a protein of ~190 kDa in native tissues including heart and brain (10, 11, 25, 26). In contrast, in heterologous expression systems, the α1C subunit has been found to be a full-length protein (e.g. Refs. 5, 15, and 16). It was not obvious if the truncation observed in the native systems was an artifact that occurred upon channel isolation or the result of a physiological processing event. However, earlier findings demonstrated that the C-terminal domain could be visualized in intact cardiac myocytes using an immunocytochemical approach (10). In addition, we found that exogenous chymotrypsin can cleave full-length expressed α1C subunits into a 190-kDa body that is very similar to the ~190-kDa fragment observed in native tissues and C-terminal fragments of 35–50 kDa (17). Interestingly, the C-terminal fragments remained associated with the membrane after cleavage (17). A proline-rich domain was identified between amino acids 1974 and 2000 and found to be important for the tethering of the C-terminal fragments to the membrane (17). Here we have presented complimentary findings that demonstrated that the C-terminal fragments associated with C-terminal-truncated channels and regulated channel activity. The domain termed CT7 was found to contain an inhibitory domain; however CT4, which contained the CT7 sequence, appeared to interact with the α1C subunit more effectively. Conceivably the difference in abilities of CT7 and CT4 to associate with the channels was due to the proline-rich domain. CT7 lacks the proline-rich domain (17), whereas CT4 contains this motif.

It appears that the C terminus of the α1C subunit is a complex and important component of this protein. The present results together with those of an earlier study (17) support the
hypothesis that the α1C subunit may undergo a physiologically important processing event in native systems and that the C-terminal fragments may remain associated with the channels to allow for channel regulation. A simplified version of our view of how the C terminus of the channel functions to regulate channel conductance is depicted in the schematic presented in Fig. 13. An extension of these conclusions is that if the endogenous C-terminal fragments do remain associated with the channels, one might predict that injection of the exogenous C-terminal peptides into cardiac myocytes would have little or no effect. Indeed, in preliminary experiments, we tested the effects of CT4 on L-type currents from adult rabbit cardiac myocytes and found that the exogenously applied CT4 did not inhibit their whole cell L-type Ca2+ current (data not shown). Further experiments will be necessary to define the point of cleavage and the nature of the C-terminal fragments in native systems.

Other important proteins involved in signaling are known to undergo regulated proteolysis (27). Examples include the sterol regulatory element-binding proteins, the amyloid precursor protein that is important in developmental signaling (27). Further experiments will be necessary to define the point of cleavage and the nature of the C-terminal fragments in native systems.

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C-terminal Fragments of the αIC(Cav1.2) Subunit Associate with and Regulate L-type Calcium Channels Containing C-terminal-truncated αIC Subunits
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