Voltage-gated Ca$_{1.2}$ channels are composed of the pore-forming $\alpha_{1C}$ and auxiliary $\beta$ and $\alpha_2\delta$ subunits. Voltage-dependent conformational rearrangements of the $\alpha_{1C}$ subunit C-tail have been implicated in Ca$^{2+}$ signal transduction. In contrast, the $\alpha_{1C}$ N-tail demonstrates limited voltage-gated mobility. We have asked whether these properties are critical for the channel function. Here we report that transient anchoring of the $\alpha_{1C}$ subunit C-tail in the plasma membrane inhibits Ca$^{2+}$-dependent and slow voltage-dependent inactivation. Both $\alpha_2\delta$ and $\beta$ subunits remain essential for the functional channel. In contrast, if $\alpha_{1C}$ subunits are expressed with $\alpha_2\delta$ but in the absence of a $\beta$ subunit, plasma membrane anchoring of the $\alpha_{1C}$ N terminus or its deletion inhibit both voltage- and Ca$^{2+}$-dependent inactivation of the current. The following findings all corroborate the importance of the $\alpha_{1C}$ N-tail/$\beta$ interaction: (i) co-expression of $\beta$ restores inactivation properties, (ii) release of the $\alpha_{1C}$ N terminus inhibits the $\beta$-deficient channel, and (iii) voltage-gated mobility of the $\alpha_{1C}$ N-tail vis à vis the plasma membrane is increased in the $\beta$-deficient (silent) channel. Together, these data argue that both the $\alpha_{1C}$ N- and C-tails have important but different roles in the voltage- and Ca$^{2+}$-dependent inactivation, as well as $\beta$ subunit modulation of the channel. The $\alpha_{1C}$ N-tail may have a role in the channel trafficking and is a target of the $\beta$ subunit modulation. The $\beta$ subunit facilitates voltage gating by competing with the N-tail and constraining its voltage-dependent rearrangements. Thus, cross-talk between the $\alpha_{1C}$ C and N termini, $\beta$ subunit, and the cytoplasmic pore region confers the multifactorial regulation of Ca$_{1.2}$ channels.

Ca$_{1.2}$ channels are known for their key role in triggering Ca$^{2+}$ signaling in a wide variety of cells. Calmodulin (CaM) regulates Ca$_{1.2}$ conductance by responding to Ca$^{2+}$ binding that shuttles it between two CaM-binding sites in the proximal half of the $\alpha_{1C}$ subunit C-terminal tail (1–4). CaM signals Ca$^{2+}$ for transcription activation (5) or Ca$^{2+}$-induced intracellular Ca$^{2+}$ release (6) by the voltage-gated rearrangement of the $\alpha_{1C}$ subunit C terminus, thus linking Ca$^{2+}$-dependent inactivation (CDI) and Ca$^{2+}$ signal transduction (7). With these voltage- and Ca$^{2+}$-gated rearrangements, the role of the Ca$_{1.2}$ cytoplasmic termini may be further defined when the $\alpha_{1C}$ subunit tails are uncoupled from the channel regulation by transient immobilization in the plasma membrane.

The association of $\alpha_{1C}$ with the auxiliary $\alpha_2\delta$ and $\beta$ subunits is important for the functional expression of Ca$_{1.2}$ channels. The cytoplasmic $\beta$ subunit binds to a conserved “u-interaction domain” in the $\alpha_{1C}$ subunit cytoplasmic linker between transmembrane repeats I and II (8, 9). The extracellular $\alpha_2$ subunit is bound via an SS bridge to its post-translationally cleaved transmembrane $\delta$ peptide (10, 11) that renders association with $\alpha_{1C}$. Both $\alpha_2\delta$ (12–14) and $\beta$ subunits (15–19) modulate the channel. In particular, $\beta$ subunits affect the time course of the Ba$^{2+}$ current decay up to 3-fold depending on the type of the $\beta$ subunit.

To study conformational rearrangements in the channel in response to depolarization, measurements of differential changes in fluorescence resonance energy transfer (FRET) between the cyan (ECFP) and yellow (EYFP) fluorescent proteins fused to the $\alpha_{1C}$ and $\beta$ subunit termini have been an effective approach. The current findings begin to specify the central features of conformational rearrangements associated with the transition of the channel from the resting (−90 mV) to the inactivated state of Ca$_{1.2}$. With the (EYFP)$_{\alpha_{2C}}$(ECFP)$_{\alpha_{1C}}$/EYFP$_{\beta_1}\alpha_\delta\beta_2$ channel as a model, FRET microscopy showed reversible voltage-gated rearrangements between the $\alpha_{1C}$-$\delta$ tails and pointed to a role for the C-terminal mobile tail in intracellular Ca$^{2+}$ signal transduction (5). Another study (20) characterized the voltage-gated rearrangements between the N-terminal tails of the $\alpha_{1C}$ and $\beta$ subunits associated with differential $\beta$ subunit modulation of inactivation and demonstrated limited rearrangements of both N-tails with regard to the plasma membrane.

To investigate further the role of voltage-gated mobility of the $\alpha_{1C}$ N-terminal tail for function of the Ca$_{1.2}$ channel, here we have investigated the effects of N-terminal deletion or plasma membrane immobilization on Ca$^{2+}$- and voltage-dependent inactivation, as well as $\beta$ subunit regulation of the channel. Most interestingly, if the $\alpha_{1C}$/Ca$^{2+}$- and voltage-dependent inactivation, as well as $\beta$ subunit regulation of the channel. Most interestingly, if the $\alpha_{1C}$/Ca$^{2+}$- and voltage-dependent inactivation, as well as $\beta$ subunit regulation of the channel.

---

*D* This work was supported by the National Institutes of Health, NIA Intramural Research Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The on-line version of this article (available at http://www.jbc.org) contains Methods, Results and Discussion, Refs. 1–7, Fig. 1, and Table 1.

‡ To whom correspondence should be addressed: Laboratory of Clinical Investigation and Cardiovascular Science, NIA, National Institutes of Health, Baltimore, Maryland 21224.

§ The abbreviations used are: CaM, calmodulin; ADSI, the annular domain sensitive to inactivation; CDI, Ca$^{2+}$-dependent inactivation; I-V, current-voltage; PH, pleckstrin homology; PIP$_2$, phosphatidylinositol bisphosphate; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; FRET, fluorescence resonance energy transfer.
integrates the β subunit and the α1C subunit C terminus in inactivation of the channel.

MATERIALS AND METHODS

Molecular Biology—Reverse transcription-PCR cloning of human hippocampal α1C subunit (see Supplemental Material) showed substantial diversity of the transcripts because of alternative splicing. The exon-22 isoform (GenBank™ accession number Z34515) of the human hippocampus α1C subunit, known as α1C,C77 (21), was selected for this study because it was identified in other human tissues and cells. (EYFP)N-α1C,C77, (PH-EYFP)N-α1C,C77, α1C,C77-(PH-ECFP)C, and (EYFP)N-PH and (EYFP)C-(PH)N-2 (22) expression plasmids (N and C indicate the N and C terminus of a subunit, respectively) were prepared in the pcDNA3 vector for eukaryotic expression as described earlier (5) by using pEYFP and pECFP vectors (Clontech). To delete the N-terminal amino acids 2–120 of the α1C,C77 subunit, the PCR product obtained by amplification of pHLC77 (21) with the sense 5′-tggatccgccacATGGTCGAATG-3′ and antisense 5′-AGCGCATGGCCCATCATCACCATCAC-3′ primers (nucleotides are shown in lowercase letters) was digested by BamHI and SgrAI and ligated at the respective sites into pcDNA3 (23). Nucleotide sequences of all PCR and ligation products were determined at the DNA sequencing facility of the University of Maryland. The βL, and αβ subunits in pcDNA3 vector were prepared as described by Soldatov et al. (24).

Transient Expression in COS1 Cells—COS1 cells were maintained at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum (Invitrogen). For transient Ca2+ channel expression, cells were plated on poly-n-lysine-coated coverslips (Mawell). The transfected cells were imaged, and the plasma membrane was labeled with the fluorescent dye 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) at room temperature (20–22 °C) using the Axophot 200B amplifier (Axon Instruments) 48–72 h after transfection. The extracellular bath solution contained the following (in mM): 110 NaCl, 20 BaCl2, 1 MgCl2, 10 glucose, 10 HEPES, adjusted to pH 7.4, with NaOH. Borosilicate glass pipettes were fire-polished and showed a typical resistance of 3–6 megohms when filled with pipette solution containing the following (in mM): 110 CsCl, 5 MgATP, 10 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, 20 tetraethylammonium, 0.2 cAMP, and 20 HEPES, adjusted to pH 7.4 with CsOH (24). Capacitance compensation and series resistance were set at 60%. Currents were sampled at 2.5–5 kHz and filtered at 1 kHz. Voltage protocols were generated, and data were digitized, recorded, and analyzed using pClamp 8.1 software (Axon Instruments). The holding potential (VH) was −90 mV, and test pulses were applied at 30-s intervals. Current–voltage (I–V) curves were obtained by step depolarizations to the indicated potentials (with 10-mV increments) in the range of 0 to −56.1 mV (Fig. 1A, inset) that, in a wide range of the current-evoking potentials, composed 35.5 ± 3.5% (n = 7) of the total Ba2+ current (from 31.4% at −10 mV to 56.1% at +50 mV). The corresponding averaged I–V relation is presented in Fig. 1D. The most prominent feature of the Ba2+ current through the channel with the plasma membrane-anchored α1C,C77 subunit C terminus was the sustained component of the current (Fig. 1D, open circles in the I–V curve) that, in a wide range of the current-evoking potentials, composed 35.5 ± 3.5% (n = 7) of the total Ba2+ current (from 31.4% at −10 mV to 56.1% at +50 mV). The sustained current was preceded by a rapidly inactivating component with the fast time constant τ of 20.8 ± 5.0 ms (at +20 mV; n = 9). Overall, the anchoring of the α1C,C77 subunit C terminus in the plasma membrane accelerated fast inactivation and inhibited slow inactivation of the Ba2+ current.

Release of the α1C,C77 subunit C-tail could be stimulated by the hydrolysis of the PH domain PI3P upon activation of phospholipase C. In previous studies, we demonstrated that activation of PI3P hydrolysis by epidermal growth factor-mediated stimulation of the co-expressed epidermal growth factor receptors helped to fully restore the CDI and voltage-dependent slow inactivation of the channel (5). Because the epidermal growth factor-mediated recovery was transient and additionally complicated by inhibition of the channel activity (27), we analyzed the effects of the co-expression of

RESULTS

Our goal in this study was to reveal the role of the α1C,C77 subunit C- and N-terminal tails in inactivation of the Ca1.2 channel and its regulation by β subunits. To simplify the interpretation of the results, in most of this series of experiments we used the protein kinase C-insensitive Ca1.2 assembled with the α1C,C77 subunit identified in human hippocampus (see Supplemental Material). Our previous experiments (5, 20, 25) provided evidence that electrophysiological properties of the channel remained essentially unaltered by the N- and/or C-terminal fusion of the green fluorescent protein analogs to the α1C,C77 subunit. Measurements of state-dependent FRET, as an indicator of the voltage-gated rearrangements of the ECFP/ EYFP-labeled channel, have demonstrated significant mobility of the α1C subunit C-terminal tail and its crucial role for Ca2+ signal transduction, contrasting with a relatively weak β subunit-dependent mobility of the α1C N terminus.

Effect of Plasma Membrane Immobilization of the α1C C Terminus on CDI, Voltage-dependent Inactivation, and Ion Selectivity of the Channel—In the first set of experiments, the C terminus of the α1C,C77 subunit was immobilized to the plasma membrane via the pleckstrin homology (PH) domain of phospholipase C61. To prepare the plasmid encoding α1C,C77-(PH-ECFP)C, the PH domain was genetically fused to the last C-terminal Leu-2138 residue of the α1C,C77 subunit, and the reading frame was completed with the ECFP-coding sequence. The β subunit-deficient α1C,C77-(ECFP)N/αβδ channel was predominantly localized in the cytoplasm and did not show substantial membrane targeting (for details, see Harry et al. (26)). The C-terminal fusion of the PH domain was sufficient to direct the plasma membrane insertion of the channel, as one can see from the distribution of the ECFP fluorescence across the cell (Fig. 1A, inset). When co-expressed in COS1 cells with the αβδ subunit but in the absence of β subunits (Fig. 1A), the α1C,C77-(PH-ECFP)C/αβδ channel generated only a minute Ba2+ current in response to a +10-mV depolarization applied from Vh = −90 mV. However, when αβδ and α1C,C77-(PH-ECFP)C were co-expressed with the βL subunit (Fig. 1B), the amplitude of the Ba2+ current increased severalfold, and the current decay exhibited a distinctly prolonged plateau at approximately half-maximum of the current. Fig. 1C shows a set of the representative traces of the Ba2+ current evoked by 600-ms test pulses in the range of 0 to +50 mV (10-mV increments) applied from Vh = −90 mV. The corresponding averaged I–V relation is presented in Fig. 1D. The most prominent feature of the Ba2+ current through the channel with the plasma membrane-anchored α1C subunit C terminus was the sustained component of the current (Fig. 1D, open circles in the I–V curve) that, in a wide range of the current-evoking potentials, composed 35.5 ± 3.5% (n = 7) of the total Ba2+ current (from 31.4% at −10 mV to 56.1% at +50 mV). The sustained current was preceded by a rapidly inactivating component with the fast time constant τ of 20.0 ± 5.0 ms (at +20 mV; n = 9). Overall, the anchoring of the α1C subunit C terminus in the plasma membrane accelerated fast inactivation and inhibited slow inactivation of the Ba2+ current.
the constitutively active mutant (Q209L) of the Ga_{q} protein that depletes the plasma membrane PIP_{2} (e.g., see Howes et al. (28)). Fig. 1E (inset) shows the fluorescence image of a COS1 cell co-expressing the α_{1C,77}-(PH-ECFP)C, α_{2δ}, and β_{1a} subunits, the Ga_{q} Q209L mutant, and representative traces of the Ba^{2+} current generated in response to step depolarizations in the range of +10 to +40 mV applied from V_{h} = −90 mV. Co-expression of the Ga_{q} Q209L mutant shifted inactivating.
viation of the channels to the normal phenotype with a prominent slow component. Single exponential fitting shows the time constant of the Ba$^{2+}$ current decay of 200 ± 38 ms (at +10 mV; n = 9) and the lack of sustained component.

The inability of the channel with the immobilized C-terminal tail to complete inactivation was corroborated by the analysis of steady-state inactivation properties (Fig. 1F). Approximately 70% of the Ba$^{2+}$ current through the α1C,77-(PH-ECFP)$_2$/α2δβ$_{1a}$ channel evoked by a +10-mV depolarization remained non-inactivated after a depolarizing prepulse in a range of −10 to +50 mV was applied from $V_h = −90$ mV prior to the +10-mV test pulse (Fig. 1F, closed circles). The release of the α1C,77 C-tail restored inactivation of the channel, as can be seen from a comparison of the Ba$^{2+}$ current decay (Fig. 1E) and steady-state inactivation curves recorded in the absence and in the presence of the constitutively active Ga$_q$ Q209L mutant (Fig. 1F).

Ca$_{1.2}$ channels classically inactivate by a combination of the voltage- and Ca$^{2+}$-dependent mechanisms. One of the main consequences of the replacement of the Ba$^{2+}$ for Ca$^{2+}$ as the charge carrier is an acceleration of the macroscopic current decay or CDI (29). However, this was not found to hold true for the C-terminal tail-anchored Ca$^{2+}$ channel. Indeed, the representative traces of the Ca$^{2+}$ current through the α1C,77-(PH-ECFP)$_2$/α2δβ$_{1a}$ channel (Fig. 1G), recorded from the same cell as Ba$^{2+}$ currents in Fig. 1C, show both inactivating and sustained components of the decay. Similar to the Ba$^{2+}$ current, the large sustained Ca$^{2+}$ current components lasted for the duration of depolarization at all indicated test pulses (+20 to +50 mV). Although the fast component of inactivation was not as prominent as in the case of the Ba$^{2+}$ current, the average fraction of the sustained currents (44.9 ± 2.3%, from 33.3% at −10 mV to 48.6% at +50 mV, Fig. 1H) was essentially the same as with Ba$^{2+}$ as the charge carrier. All these features of the C-terminal tail-anchored α1C,77-(PH-ECFP)$_2$ channel closely matched the phenotype of the α1C,88,IV channel, which has the slow inactivation mechanism inhibited by the specific mutation in the cytoplasmic pore region (30).

The I-V relations for the Ba$^{2+}$- and Ca$^{2+}$-conducting α1C,77-(PH-ECFP)$_2$ channel show a number of common patterns (Fig. 1, D and H). Immobilization of the α1C,77 subunit C-terminal tail caused a shift in activation of ion conductance by 10–15 mV to more positive potentials. Currents reached the peak of the I-V relationship at 20 (Ca$^{2+}$) or +30 mV (Ba$^{2+}$), exhibiting a 10–20-mV shift of the maximum toward more positive voltages as compared with the α1C,77-(ECFP)$_2$/α2δβ$_{1a}$ channel. The apparent reversal potential was also notably changed. Both the Ba$^{2+}$ and Ca$^{2+}$ currents reversed direction at much higher voltages than in the wild-type channel. Although this effect was not investigated in detail, it may be due to altered ion selectivity and/or decreased permeability to Ca$^{2+}$ ions (introduced in electrodes) in the outward direction that contributed significantly to the apparent reversal potential of the wild-type channel (≈ +65 mV) (31). Release of the α1C,77-(PH-ECFP)$_2$ subunit C-terminal tail by co-expression of the Ga$_q$ Q209L mutant reversed these changes in the I-V relationship to parameters that are more characteristic for the α1C,77-(ECFP)$_2$/α2δβ$_{1a}$ channel, including ion selectivity and the peak current voltage (Fig. 1, D, filled squares). The permeability of the wild type α1C,77 Channel to Ba$^{2+}$ is on average 2.8 times greater than that of the Ca$^{2+}$ ions (24, 30). Immobilization of the α1C subunit C-terminal tail may affect the ion conductance of the channel. Taken together, these data support the model (7) that links CDI and slow inactivation of the Ca$_{1.2}$ channel to specific folding of the α1C subunit C terminus vis à vis the cytoplasmic pore region.

β Subunit Facilitation of the Ca$_{1.2}$ Channel Gating Is Revealed by Immobilization of the α1C Subunit N-terminal Tail—In the second set of experiments, the N-terminal tail of the (EYFP)$_{N}$−α1C,77 subunit was anchored to the plasma membrane via the PH domain. The (PH-EYFP)$_{N}$−α1C,77 channel was expressed in COS1 cells in different combinations with auxiliary β$_{1a}$ and α$_2$δ subunits. Fig. 2 shows a collection of representative traces of the Ba$^{2+}$ current elicited by depolarization to +10 mV from $V_h = −90$ mV in a set of COS1 cells of approximately similar size. The insets in Fig. 2 are fluorescent images of the expressing cells showing subcellular localization of the EYFP-tagged α1C subunits. Confirming earlier data (32, 33), COS1 cells did not show appreciable endogenous expression of Ca$_{1.2}$ (Fig. 2A). Transfection of COS1 cells with a mixture of cDNAs coding for the (EYFP)$_{N}$−α1C,77, β$_{1a}$ and α$_2$δ subunits (Fig. 2B) renders the current with characteristics (Fig. 3, curves 3) closely resembling those of the wild-type channel (24). Anchoring of the α1C subunit N terminus by co-expression of (PH-EYFP)$_{N}$−α1C,77 with the β$_{1a}$ and α$_2$δ subunits (Fig. 2C) accelerated the kinetics of inactivation of the Ba$^{2+}$ current by −15% (5). An acceleration of inactivation was also seen when the PH domain was separately co-expressed with the (EYFP)$_{N}$−α1C,77, β$_{1a}$, and α$_2$δ subunits (Fig. 2D). Making no assumptions about the nature of these variations, we investigated the effect of the deletion of the β$_{1a}$ subunit from the expressed constituents of the channel.

When (EYFP)$_{N}$−α1C,77 was expressed alone, the fluorescent-tagged channel protein was diffusely distributed over the cytoplasm and did not generate measurable voltage-gated current (Fig. 2E). The N-terminal fusion of the PH domain caused robust surface membrane targeting by the labeled (PH-EYFP)$_{N}$−α1C,77 protein, but the channel remained essentially silent (Fig. 2F). A similar result was obtained when α$_2$δ was co-expressed with the (EYFP)$_{N}$−α1C,77 subunit (Fig. 2G, note poor membrane targeting of the channel complex). However, the membrane anchoring of the α1C subunit N-terminal tail by co-expression of the (PH-EYFP)$_{N}$−α1C,77 and α$_2$δ subunits stimulated membrane targeting of the β-deficient channel that generated large slowly activating and non-inactivating Ba$^{2+}$ current in response to depolarization (Fig. 2H). This result is consistent with the idea that α1C and α$_2$δ are sufficient for the expression of a conducting L-type Ca$^{2+}$ channel if the N-terminal tail of the α1C subunit is immobilized by the plasma membrane anchoring. To find whether or not release of the PH-tagged N-terminal tail would compromise the channel activity in the β-subunit-deficient channel, we investigated the effect of co-expression of the constitutively active Q209L mutant of Ga$_q$ A Ga$_q$-mediated depletion of PIP$_2$ in the plasma membrane did abolish the plasma membrane targeting by inhibiting the immobilization of the α1C,77 subunit N-terminal tail, and precluded expression of the conducting channel (Fig. 2I). Together, these data suggest that the α1C N-terminal tail may act as a silencer of the channel voltage-gated conductance and block it in the absence of the β subunit. Results obtained with the α1C subunit having a genetically immobilized N-terminal tail further corroborate this suggestion by demonstrating robust current of the β subunit-deficient Ca$_{1.2}$ channel composed of the (PH-EYFP)$_{N}$−α1C,77 and α$_2$δ subunits.

Activation and Inactivation Properties of the (PH-EYFP)$_{N}$−α1C,77/α2δ Channel—The effects of the α1C subunit N-tail immobilization in the plasma membrane on electrophysiological properties of the channel are presented in Fig. 3. For comparison with the channel that has the α1C subunit N-terminal tail
EYFP-tagged but not anchored, Fig. 3, A–C, includes data for Ba\(^{2+}\) currents through the (EYFP)\(_{\alpha_{1C,77}N}\)β/αδ channel (curves 3). Fig. 3A shows superimposed traces of Ba\(^{2+}\) currents (normalized to the same amplitude) through the (PH-EYFP)\(_{\alpha_{1C,77}N}\)β/αδ channel (trace 1), (PH-EYFP)\(_{\alpha_{1C,77}N}\)β/αδ channel (trace 2), and the control channel (trace 3). When co-expressed with the β\(_1\) and αδ subunits, the (PH-EYFP)\(_{\alpha_{1C,77}N}\) channel showed notable acceleration of inactivation (Fig. 3A, trace 1) compared with the control. However, expression of the same channel in the absence of the β subunit (Fig. 3A, trace 2) significantly delayed activation and inhibited inactivation of the current. A single-exponential fit of the activation time course (+20 mV) showed a substantial increase in the time constant of activation (τ\(_a\)) of the Ba\(^{2+}\) current through the β-deficient (PH-EYFP)\(_{\alpha_{1C,77}N}\)β/αδ channel (34.0 ± 10.5 ms, n = 13) as compared with the (PH-EYFP)\(_{\alpha_{1C,77}N}\)β/αδ channel (τ\(_a\) = 2.4 ± 0.3 ms; n = 6; p < 0.005). The respective I-V relationships are shown in Fig. 3B. The voltage-dependent characteristics, obtained from the fit of the I-V curve for the control (PH-EYFP)\(_{\alpha_{1C,77}N}\)β/αδ channel, were typical for the L-type Ca\(^{2+}\) channel current, including E\(_{rev}\) = 60 ± 2 mV, V\(_{1/2}\) = 0.02 ± 0.05 mV, and k\(_{rev}\) = -7.8 ± 0.5 mV (n = 6). The plasma membrane immobilization of the α\(_{1C,77}\) subunit N-terminal tail shifted the voltage dependence of activation (V\(_{1/2}\)) to more negative potentials by -15.5 ± 2.4 mV (β\(_1\)αδ, n = 6) and -23 ± 4.5 mV (β\(_1\)αδ, n = 13). The threshold of activation of the Ba\(^{2+}\) current was shifted by -10 mV toward more negative voltages in the β-deficient channel (Fig. 3B, filled circles) as compared with the (PH-EYFP)\(_{\alpha_{1C,77}N}\)β/αδ channel (open circles). In the β-deficient channel, the voltage that elicited the maximum Ba\(^{2+}\) current (+10 mV) was shifted by -10 mV to more positive potentials. However, the most notable change was the increase of the apparent reversal potential in both (PH-EYFP)\(_{\alpha_{1C,77}N}\)β/αδ channels. A similar effect was also induced by immobilization of the α\(_{1C}\) subunit C-terminal tail (Fig. 1) and may be a result of altered ion selectivity.

To characterize further the inactivation characteristics of the Ba\(^{2+}\) current through the (PH-EYFP)\(_{\alpha_{1C,77}N}\)β/αδ channels, we measured steady-state inactivation curves by using a two-step voltage clamp protocol (Fig. 3C). Steady-state inactivation properties point to differences in voltage dependence of inactivation of the Ba\(^{2+}\) current as compared with the control (PH-EYFP)\(_{\alpha_{1C,77}N}\)β/αδ channel (Fig. 3C, curve 1). Voltage at the half-maximum of inactivation was shifted from -7.9 ± 0.7 mV (n = 6) in control to -15.9 ± 3.4 mV (n = 3) in the (PH-EYFP)\(_{\alpha_{1C,77}N}\)β/αδ channel (Fig. 3C, curve 3). Although the voltage dependence of availability (20.1 ± 1.5%) was not significantly changed by the plasma membrane immobilization of the α\(_{1C}\) subunit N-terminal tail (21.0 ± 5.9%), the slope β of the steady-state inactivation curve (fitted by the Boltzmann equation) decreased from 6.3 ± 0.6 to 13.5 ± 3.8 (p < 0.05). Thus, cooperativity in voltage gating leading to inactivation is significantly affected by the plasma membrane immobilization of the α\(_{1C}\) subunit N-terminal tail. Steady-state inactivation analysis also confirmed that the voltage dependence of inactivation of the β subunit-deficient channel is inhibited in the range of conditioning pulses of up to +60 mV (Fig. 3C, curve 2).

The single-channel analysis corroborated the macroscopic data (Fig. 4, A and B). Recordings of the Ba\(^{2+}\) current in the cell-attached configuration (110 mm Ba\(^{2+}\)) revealed rarely occurring activations of the (PH-EYFP)\(_{\alpha_{1C,77}N}\)β/αδ channel that were characterized by low probabilities of both opening and closing (Fig. 4B). Openings of the channel were preceded by a large number of blank traces. The loss of inactivation of
Regulation of the Ca_{1.2} Channel by the α_{1C} Subunit Tails

The characteristic, slowly activating and non-inactivating phenotype of the β subunit-deficient (PH-EYFP)_{N-α_{1C,77}/α_2δ} channel was also found to hold true with Ca^{2+} as the charge carrier. Fig. 5A shows superimposed traces of the Ca^{2+} current evoked by depolarization in the range of -20 to +50 mV applied from $V_h = -90$ mV. A remarkable feature of the β-deficient channel is the striking similarity in the properties of the Ba^{2+} and Ca^{2+} currents. A single-exponential fit of the activation time course (+20 mV) shows a marked increase ($p < 0.05$) in the time constant of activation of the Ca^{2+} current through the β-deficient (PH-EYFP)_{N-α_{1C,77}/α_2δ} channel ($\tau_a = 69.0 \pm 12.5$ ms, $n = 32$) as compared with the (PH-EYFP)_{N-α_{1C,77}/β_1/α_2δ} channel ($\tau_a = 2.0 \pm 0.2$ ms; $n = 5$; Fig. 5C). These data are not significantly different (unpaired t test) from those obtained for the Ba^{2+} current (see above). The β subunit-deficient channel exhibits unusually high Ca^{2+} current amplitude but did not reveal appreciable decay of the Ca^{2+} current even when the test pulse duration was prolonged to 30 s (Fig. 5B). These data indicate that CDI was lost by the β subunit-deficient Ca_{1.2} channel when its α_{1C} subunit N-tail was immobilized in the plasma membrane. Release of the N terminus of the (PH-EYFP)_{N-α_{1C,77}} subunit by the co-expression of a constitutively active Q209L mutant of G_{α}, to activate PIP_{2} hydrolysis (Fig. 5D) sharply reduced the size of the Ca^{2+} current through the β subunit-deficient channel in response to a +20-mV depolarization that corresponds to other controls discussed above (Fig. 2, G and I). A small residual non-inactivating current with the average amplitude of 30 pA may be due to incomplete PIP_{2} hydrolysis rendering plasma membrane immobilization of the α_{1C} subunit N-tail in a small fraction of the channels (see also Fig. 2F). Taken together, these data suggest that inactivation by both the voltage-dependent and CDI mechanisms is missing in the β subunit-deficient (PH-EYFP)_{N-α_{1C,77}/α_2δ} channel.

Effect of the α_{1C} Subunit N-terminal Deletion on the β Subunit Modulation of the Ca_{2+} Channel Gating—We have next explored another way of uncoupling the α_{1C} subunit N-tail from its apparent silencing function by a genetically encoded deletion of 120 out of a total 124 N-terminal amino acids of α_{1C,77} (ΔN-α_{1C,77}). Shistik et al. (34) made a similar attempt in Xenopus oocytes by expressing a rabbit cardiac α_{1C} where all but 15 N-terminal tail amino acids were deleted. However, because Xenopus oocytes are known to express endogenous Ca^{2+} channels (35–38), there was ambiguity in their findings. Interpretation was also obscured by the continuous application of 1 μM (-)-BayK8644 to artificially increase the amplitude of the single channel Ba^{2+} current. More substantial deletion of the α_{1C} subunit N-terminal tail as well as the use of COS1 cells, free of endogenous Ca^{2+} channel subunits, helped us to eliminate these ambiguities. Fig. 6 shows Ca^{2+} channel activity of the ΔN-α_{1C,77}/α_2δ channel assembled of (A) or without (B) the β subunit. As was observed with the full size α_{1C,77} (for details see Refs. 20 and 26), the co-expression of ΔN-α_{1C,77} and α_2δ increased the plasma membrane targeting by the (EYFP)_{N-β_1α_2} subunit (Fig. 6A, panel a). The surface membrane targeting by the labeled ΔN-α_{1C,77}/EYFP_{K} subunit (and the (EYFP)_{N-α_2δ}; Fig. 6B, panel a) was prominent independently on the presence of the β subunit (Fig. 6, A and B, panel b). The presence of β was not crucial for the functional expression of the ΔN-α_{1C,77}/α_2δ channels. In both channels, Ca^{2+} currents were activated by stepwise depolarization from $V_h = -90$ mV (shown are traces recorded in response to +20-mV test pulses). This result supports the data on the α_{1C,77} N-tail anchoring that a β subunit is not essential for the expression of an active conducting channel if the N-terminal tail of the α_{1C} subunit is uncou-
fig. 4. Effects of the α1C,77 subunit N terminus uncoupling by anchoring or deletion on single channel Ba^{2+} currents through the β subunit-deficient Ca^{2+} channels expressed in COS1 cells. Ba^{2+} currents were measured in 110 mM Ba^{2+} and high K^+ solution at a stimulation rate of 0.1 Hz. A and B, effect of anchoring of the α1C,77 subunit N terminal in the plasma membrane with (A) and without β1a (B) subunit expressed. (PH-EYFP)β-Δα1C,77/α2δ channel current was activated by a test pulse to 0 mV. Insets show detailed single channel activity. Single channel averages are depicted in gray under the original traces in A and B with exponential fit and mean level, respectively, (n = 74 and n = 34). Respective first latency histograms are also shown with their exponential fits. C and D, effect of the α1C,77 subunit N terminal deletion with (C) and without β1a (D) subunit expressed. The Δ_N^-α1C,77/α2δ channel current was activated by a test pulse to -10 mV. Length of bursts was 234.7 ± 31.6 ms, (n = 40) (C); and 361.9 ± 70.3 ms, (n = 23, p < 0.05) (D).

Regulation of the Cav1.2 Channel by the α_1C Subunit Tails

pled from the channel regulation. Although the β subunit was crucial for inactivation of the (PH-EYFP)β-Δα1C,77/α2δ channel (Fig. 2C), this effect was essentially missing in the Δ_N^-α1C,77/α2δ channel (Fig. 6A). The Ca^{2+} current evoked by the +20-mV step depolarization had rather small amplitude and did not show accelerated decay that would be characteristic for CDI. This result indicates that the functional uncoupling of the α1C subunit N-terminal tail through the plasma membrane anchoring or deletion influences the interaction with the β subunit in different ways.

Similar to the β-deficient (PH-EYFP)β^-Δα1C,77/α2δ channel that shows non-inactivating Ca^{2+} currents (Fig. 5B), inactivation of the Δ_N^-α1C,77/α2δβ^-β1a channels was impaired (Fig. 6, A and B). In addition, removal of the β subunit from the channel
Fig. 5. Lack of inactivation of the $\mathrm{Ca}^{2+}$ current through the $\beta$ subunit-deficient $\mathrm{Ca}_{1.2}$ channel with the N-terminal tail of the $\alpha_{1C}$ subunit immobilized in the plasma membrane. The (PH-EYFP)$_{\alpha_{1C,77}}$ and $\alpha_\delta$ subunits were co-expressed in COS1 cells in the absence (A–C) or in the presence (D) of the constitutively active Go$_{q}$ Q209L mutant to enforce (A and B) or to prevent (D) the PH domain-mediated plasma membrane-anchoring of the $\alpha_{1C,77}$ subunit N-tail. In control (C), co-expression of the (PH-EYFP)$_{\alpha_{1C,77}}$ and $\alpha_\delta$ subunits with $\beta_{1a}$ restored inactivation. $\mathrm{Ca}^{2+}$ currents were elicited by a series of 600-ms test pulses to the indicated voltages in the range of $\pm 20$ to $\pm 50$ mV (A) or by a $\pm 20$-mV depolarization for 30 s (B) or 600 ms (C and D) applied from $V_{h} = -90$ mV.

complex significantly delayed activation of the $\mathrm{Ca}^{2+}$ current (Fig. 6B). However, analysis of the respective I-V relations (Fig. 6C) showed that the voltage sensors responsible for activation of the $\Delta_{V}^{\alpha_{1C,77}/\alpha_\delta} - \beta_{1a}$ channels were not strongly affected by the removal of the $\beta$ subunit from the oligomeric complex, despite other signature changes also seen with the N-tail-anchored $\alpha_{1C,77}$ channel (Fig. 5A), including broadening of the I-V curves and a shift of the peak currents to more positive potentials (Fig. 6C). Single channel recordings (Fig. 4D) revealed a relatively large number of empty swipes corresponding to low probability of opening of the $\beta$ subunit-deficient $\Delta_{V}^{\alpha_{1C,77}/\alpha_\delta}$ channel. First latencies were significantly shorter in the presence of the $\beta$ subunit, $\tau = 48.5$ ms (Fig. 4C), than without it, $\tau = 139.4$ ms (Fig. 4D). However, the important difference with the effect of the transient anchoring of the $\alpha_{1C}$ subunit N-terminal tail (Fig. 4, A and B) is clearly visible; the N-tail uncoupling through a deletion essentially stabilized the open state of the $\beta$ subunit-deficient channel, which showed long opening of a single $\mathrm{Ca}^{2+}$ channel activity during long lasting depolarizations (Fig. 4D). With either type of uncoupling of the $\alpha_{1C}$ subunit N-terminal tail, a delay in the activation of the whole-cell current appears to be associated with prolongation of the first latency.

Effect of the $\beta$ Subunit on the Voltage-gated Mobility of the $\alpha_{1C}$ Subunit N-terminal Tail—Finally, we assessed whether the $\beta$ subunit affects the voltage-gated mobility of the $\alpha_{1C}$ subunit N-terminal tail. The (EYFP)$_{\alpha_{1C,77}}/\alpha_\delta$ channel was co-expressed with the (ECFP)$_N$-PH in the absence (Fig. 7A) or in the presence (Fig. 7B) of the $\beta_{1a}$ subunit (for arrangement of fluorophores and subunits, see panels a). In this experiment, the EYFP-labeled N-terminal tail of the $\alpha_{1C,77}$ subunit reports its state-dependent position to the FRET partner, ECFP, fused to the plasma membrane-trapped PH domain. FRET was confined to the plasma membrane region (Fig. 7, yellow boxes in panels a) and was set to be recorded within the time windows, marked by black or red bars under the actual Ba$^{2+}$ current traces on panels c, simultaneously with acquisition of the currents. An image recorded at the resting state ($\sim 90$ mV, Fig. 7, black bars corresponding to panels d) was followed by an image recorded at the end of a $\pm 20$-mV depolarization test pulse (red bars corresponding to panels e). One can see that independent of the presence of the $\beta_{1a}$ subunit, a corrected FRET signal of different intensity was observed in Fig. 7, panels d and e.

Fig. 6. Effect of the $\beta$ subunit on $\mathrm{Ca}^{2+}$ currents through the $\mathrm{Ca}_{1.2}$ channel with a genetically deleted N terminus of the $\alpha_{1C}$ subunit. The $\Delta_{V}^{\alpha_{1C,77}/\alpha_\delta}$ and $\alpha_\delta$ subunits were co-expressed in COS1 cells in the presence (A) or in the absence (B) of the $\beta_{1a}$ subunit. $\mathrm{Ca}^{2+}$ currents were evoked by a $\pm 10$-mV test pulse applied from $V_{h} = -90$ mV. C, the averaged normalized I-V relationships of the $\mathrm{Ca}^{2+}$ current through the $\Delta_{V}^{\alpha_{1C,77}/\alpha_\delta}$ channel in the presence (filled circles, $n = 11$) or absence (open circles, $n = 7$) of the $\beta_{1a}$ subunit. Currents were measured with 30-s intervals between 0.6-s test pulses in the range of $-60$ to $+50$ mV applied with $10$-mV increments from $V_{h} = -90$ mV. Fluorescent images were recorded with (ECFP)$_N$-$\beta_{1a}$ (A, panel a), $\Delta_{V}^{\alpha_{1C,77}/(ECFP)_N}$ (A and B, panels b), and (ECFP)$_N$-$\alpha_\delta$ (B, panel a) that retain full functional activity (for details see Ref. 20). Scale bars, 3 $\mu$m.

Rotations (Fig. 7, panels f) between the two consecutive images recorded at $-90$ and $+20$ mV reflect voltage-dependent conformational rearrangements of the N-tail fluorophore fused to the $\alpha_{1C,77}$ subunit with regard to the relatively small membrane-trapped PH domain probe (5). In the presence of the $\beta_{1a}$ subunit (Fig. 7B), the channel generated a Ba$^{2+}$ current in response to a $\pm 20$-mV depolarization ($V_{h} = -90$ mV) that showed complete inactivation within 500 ms (Fig. 7B, panel c). Thus, the $\pm 20$-mV corrected FRET image recorded at the end of the
600-ms depolarizing pulse (Fig. 7B, panel e) corresponds to a predominantly inactivated state of the channel. However, the ratio (Fig. 7B, panel f) shows very little if any change in corrected FRET between \( \) and \( \) at the time windows marked below the current trace by black and red bars, respectively, shows a substantial (\( p < 0.005 \)) differential FRET signal generated in the plasma membrane by voltage-gated rearrangement of the \( \) subunit N-terminal tail.

**DISCUSSION**

\( \text{Ca}_{1.2} \) channels play an important role in initiation of \( \text{Ca}^{2+} \) signaling in many cells, including neurons (39). Here we have investigated differential roles of the C- and N-terminal tails of one of the neuronal “short” (exon 1) \( \alpha_{1C} \) isoforms in voltage-, \( \text{Ca}^{2+} \)-, and \( \beta \) subunit-dependent regulation of the \( \text{Ca}_{1.2} \) channel.

Our study is an important step forward in understanding
Regulation of the Ca$_{1.2}$ Channel by the $\alpha_{1C}$ Subunit Tails

Ca$_{1.2}$ channel regulation as a multifactorial process. Here for the first time we have analyzed differential regulation of the Ca$^{2+}$ channel by the C- and N-terminal tails of the $\alpha_{1C}$ subunit with simultaneous assessment of the role of $\beta$ subunits. To deconvolute the contribution of these parts, transient anchoring to the plasma membrane via the PH domain fused to the $\alpha_{1C}$ subunit tails has been used to uncouple the $\alpha_{1C}$ tails from the channel regulation. To assess the potential interplay between the tails and a $\beta$ subunit, we compared properties of the channel expressed in the absence and in the presence of $\beta$. The accumulated body of data shows that inactivation of the Ca$_{1.2}$ channel is mediated by a combinatorial input of these distinct parts, in which CDI is essentially additive to voltage-dependent inactivation.

Voltage-dependent slow inactivation and CDI were previously co-identified with the calmodulin-binding regions in the middle part of the $\alpha_{1C}$ subunit C-tail (for review see Ref. 40). A recent picture (41) still includes an EF hand-like motif of the C-tail proximal region, although involvement of this motif in CDI has been contested by experimental evidence (29). Our findings show that CDI is not mediated solely by determinants of the $\alpha_{1C}$ C-terminal tail. Investigation of other cytoplasmic constituents of the channel complex have been crucial in identifying the roles of the N-tail, the cytoplasmic pore region of $\alpha_{1C}$, and $\beta$ subunits for inactivation and in understanding how slow and fast voltage-dependent inactivation and CDI evolve from their interplay. A distinct advantage of COS1 cells lacking endogenous Ca$^{2+}$ channel subunits (32) over other expression systems, such as HEK293 cells or Xenopus oocytes, permits an unambiguous interpretation of our data on $\beta$ subunit modulation of the channel. Because no appreciable Ca$^{2+}$ channel activity was exhibited by the $\alpha_{1C,77}/\alpha_{2\delta}$ channel (Fig. 2G), unless a $\beta$ subunit was co-expressed, we define the $\beta$ subunit modulation as a facilitation of voltage gating of the channel. Recognizing that the kinetics of inactivation of the Ba$^{2+}$ current through the “wild-type” $\alpha_{1C,77}/\beta_1/\alpha_{2\delta}$ channel is best fit by a sum of two (fast and slow) exponentials, the respective fractional components of the Ba$^{2+}$ current measured at the peak of I-V curves were analyzed to assess the voltage-dependent fast and slow inactivation mechanisms.

We have shown previously that four highly conserved amino acids of the transmembrane segment S6, constituting the cytoplasmic end of the pore, jointly form the ADSI. Their simultaneous mutation (S405I in IS6, A752T in IIIS6, V1165T in IIIIS6, and 11475T in IVS6) generates the $\alpha_{1C,IS,IV}$ channel, which does not show the slow inactivation, CDI, or differential $\beta$ subunit modulation (30). Our findings have shown that the $\alpha_{1C}$ subunit C-terminal tail is subject to voltage-gated conformational rearrangements that specifically deliver Ca$^{2+}$ signaling to downstream targets involved in cAMP-response element-binding protein-dependent transcription activation (5). The plasma membrane anchoring of the C-tail interrupts Ca$^{2+}$ signal transduction despite robust Ca$^{2+}$ current through the channel. Thus, a specific position of the C-terminal tail vis à vis the polypeptide packing of the cytoplasmic channel constituents (including the $\alpha_{1C}$ subunit tails and a $\beta$ subunit) is crucial for CDI and slow voltage-dependent inactivation.

We now find that plasma membrane immobilization of the $\alpha_{1C,77}$ C-terminal tail alters inactivation of the channel similar to the mutation of the ADSI (Fig. 1). An interesting distinction from the electrophysiological phenotype of the $\alpha_{1C,IS,IV}$ channel, however, is a shift of the I-V relationship to more positive potentials. Because these dramatic changes do not involve structural alterations of the ADSI or the calmodulin-binding regions, and are completely reversible upon the release of the $\alpha_{1C,77}$(PH-ECFP)$_1$ subunit C-tail, stimulated by PIP$_2$ hydrolysis, it is reasonable to assume that the plasma membrane anchoring of the $\alpha_{1C}$ C-terminal tail interferes with the functional folding of the cytoplasmic polypeptide bundle in the pore region, including ADSI. This may directly affect the pore-forming transmembrane segment IVS6, connected to the $\alpha_{1C}$ C-tail, and, indirectly, the ensemble of the pore conformations defining the ion selectivity of the channel.

A very different role was found for the $\alpha_{1C}$ subunit N-terminal tail. FRET microscopy combined with the patch clamp (20) revealed limited voltage-dependent mobility of the $\alpha_{1C}$ N terminus in relationship with the plasma membrane (Fig. 7B). This may correspond to the restricted local dynamics of the adjacent S1 segment observed in other channels (42). Here we find that in the $\beta$ subunit-deficient (silent) channel, voltage-gated conformational rearrangements of the $\alpha_{1C}$ subunit N terminus vis à vis the plasma membrane are significantly increased (Fig. 7B). The functional importance of the conformational “rigidity” to voltage gating conferred by a $\beta$ subunit to the $\alpha_{1C}$ subunit N terminus was confirmed here by anchoring of the N-tail to the plasma membrane, stabilized by the fused PH domain.

The $\beta$ subunit confers conformational rigidity to the $\alpha_{1C}$ subunit N-tail in a manner that facilitates the channel response to voltage gating. We have found that the plasma membrane-anchoring of the $\alpha_{1C}$ subunit N terminus, in the absence of $\beta$, completely inhibited inactivation of the Ba$^{2+}$ (or Ca$^{2+}$) current. At the same time, activation of the channel current in response to depolarization becomes slow independently of the charge carrier (Fig. 2H). The single channel study pointed to low probability of both opening and closing of the channel (Fig. 4B). Uncoupling of the N-terminal tail by its deletion from the $\alpha_{1C}$ subunit also inhibited inactivation of the channel (Fig. 7). However, in this case retardation of the channel activation was enhanced to a greater extent, probably because of long lasting episodes of rare openings (Fig. 4D). Release of the plasma membrane-anchored $\alpha_{1C}$ N terminus essentially blocked the channel current (Fig. 2I), leaving only some residual Ca$^{2+}$ channel activity that may be generated by the remaining small fraction of channels with immobilized $\alpha_{1C}$ N-tails. Overall, these data correspond to the result obtained with the $\beta$-deficient Ca$_{1.2}$ channel (Fig. 2G). Conversely, co-expression of $\beta$ with $\alpha_{1C}$ subunits re-established both the voltage- and Ca$^{2+}$-dependent inactivation of the channels despite uncoupling of the $\alpha_{1C}$ subunit N-terminal tail from the regulation by its plasma membrane anchoring. These observations shed new light on the role of the $\beta$ subunit. It appears that there is a $\beta$ subunit-dependent desensitization of the $\alpha_{1C}$ subunit N-tail to voltage, which is essential for the fast activation, CDI, and voltage-dependent inactivation of the channel.

Our data support the conclusion of Dascal and co-workers (34) that $\beta$ subunits prevent inhibition of the channel by the N-tail. The results of our work demonstrate that the N-terminal tail of the $\alpha_{1C}$ subunit is a channel silencer, competing with the $\beta$ subunit facilitation of the channel gating. This may account for CDI and voltage-dependent inactivation and activation of the current.

Our findings add to the understanding of the $\beta$ subunit regulation of the Ca$_{1.2}$ channel in a number of ways. The “chaperon” hypothesis implies that $\beta$ subunits ease functional expression of the Ca$_{1.2}$ channel by binding to the $\alpha$-interaction domain resulting in inhibition of an endoplasmic reticulum retention signal of the $\alpha_{1C}$ subunit (43). The present study does not support this view. In COS1 cells, free of endogenous Ca$^{2+}$ channel subunits, an uncoupling of the $\alpha_{1C}$ subunit N-terminal tail (by membrane anchoring or deletion) completely eliminates the requirement of $\beta$ subunits.
for the robust expression of functional Ca$_{1.2}$ channels, but co-expression of α$_{0.0}$ is critical.

Recent investigation of the human cardiac short β$_{2f}$ and β$_{2g}$ subunits (26) identified the C-terminal 153-amino acid sequence as essential for the functional modulation of the channel and interaction with the α$_{1C}$ subunit. This region includes Ser-574 that is subject to phosphorylation by phosphatidylinositol 3-kinase as necessary and sufficient to promote Ca$^{2+}$ channel trafficking to the plasma membrane (44). Although the mechanism of this effect is unknown, our data put the α$_{1C}$ subunit N-terminal tail in play as a possible determinant of the channel trafficking and a target of the β subunit modulation.

Thus a new picture of the β subunit modulation of the Ca$_{1.2}$ channel has emerged (Fig. 8), showing how β acts as a “molecular wedge” that prevents the N terminus of the α$_{1C}$ subunit from blocking the pore. Dependence of CDI on interaction between the α$_{1C}$ subunit N-tail and the β subunit led us to a hypothesis that the β subunit and the α$_{1C}$ C-tail may have an integrating role for the cytoplasmic polypeptide packing underlying the pore.

Ca$_{1.2}$ channels couple membrane depolarization to distinct neuronal functions associated with regulation of exocytosis, gene expression, synaptic plasticity, cell survival, and other processes. Among the ensemble of mutually coordinated determinants of slow inactivation identified in our study, the α$_{1C}$ subunit C-terminal tail and ADSI are directly involved in this coupling, whereas the crucial correlates provided by α$_{1C}$ N terminus and β subunits may add more specialization via genetic variation and alternative splicing in neuronal cells (see Supplemental Materials and Ref. 26). Whether neuronal specialization evolves from such variations is an interesting question to be addressed.

Acknowledgments—We thank Mike Shi and Larissa Maltseva for technical help with electrophysiology and Yasir Kazmi for help with fluorescent images. We also thank Drs. Mark Mattson and Katsutoshi Furukawa for critically reading the manuscript.

REFERENCES
1. Zühlke, R. D., Pitt, G. S., Deisseroth, K., Tsien, R. W., and Reuter, H. (1999) Nature 399, 159–162
2. Pate, P., Mocha-Morales, J., Wu, Y., Zhang, J.-Z., Rodney, G. G., Serysheva, I. I., Williams, B. Y., Anderson, M. E., and Hamilton, S. L. (2000) J. Biol. Chem. 275, 39786–39792
3. Romanin, C., Gamsjäger, R., Kahr, H., Schaufler, D., Carleson, O., Abernethy, D. R., and Soldatov, N. M. (2000) FEBS Lett. 487, 301–306
4. Dzhura, I., Wu, Y., Zhang, R., Colbran, R. J., Hamilton, S. L., and Anderson, M. E. (2003) J. Physiol. (Lond.) 550, 731–738
5. Kóbirska, E., Schwartz, E., Abernethy, D. R., and Soldatov, N. M. (2003) J. Biol. Chem. 278, 5021–5028
6. Woo, S.-H., Soldatov, N. M., and Morad, M. (2003) J. Physiol. (Lond.) 552, 437–447
7. Soldatov, N. M. (2003) Trends Pharmacol. Sci. 24, 167–171
8. Pragnell, M., De Waard, M., Mori, Y., Tanabe, T., Snutch, T. P., and Campbell, K. P. (1994) Nature 368, 67–70
9. De Waard, M., Pragnell, M., and Campbell, K. P. (1994) Neuron 13, 495–503
10. De Jongh, R. S., Warner, C., and Catterall, W. A. (1990) J. Biol. Chem. 265, 14738–14741
11. Jay, S. D., Sharp, A. H., Kahl, S. D., Vedvick, T. S., Harpold, M. M., and Campbell, K. P. (1991) J. Biol. Chem. 266, 5287–5293

FIG. 8. Hypothetical molecular arrangement of the identified regulatory determinants of the Ca$_{1.2}$ channel in the resting (−90 mV) and inactivated (+20 mV) states. Shown are multifactorial molecular correlates of the Ca$_{1.2}$ channel regulation assembled in a cytoplasmic polypeptide packing (gray area) underlying the pore. The correlates include the following: (i) the ADSI as a combination of four amino acids (boldface letters) at the cytoplasmic end of transmembrane segments S6; (ii) a β subunit, associated, via its C-terminal (dark) region (26), with the linker between the repeats I and II of α$_{1C}$. Variable proximal region of the β subunit (striped) is not essential for the α$_{1C}$-β interaction (26) but may contribute to differential modulation of Ca$_{1.2}$ inactivation (20). The N termini of the α$_{1C}$ and β subunits are subject to voltage-gated rearrangements (double arrows) associated with differential β subunits modulation of slow inactivation; (iii) the α$_{1C}$ subunit N-terminal that is blocked by β from inhibiting the channel. The N-tails of both the α$_{1C}$ and β subunits do not show voltage-dependent conformational rearrangements vis-à-vis the plasma membrane unless the slow inactivation is inhibited by ADSI mutation (20); (iv) the α$_{1C}$ subunit C-terminal tail with the locus LA (amino acids 1571–1599) of apo-CaM binding (2, 3), and a post-LA (IQ) site of Ca$^{2+}$-CaM binding (1). CaM is involved in CDI as an important stabilizing component of this packing, because the Ca$^{2+}$-free CaM may cross-link the LA site to the interior of the packing, where it is hidden from the cytosolic Ca$^{2+}$ and available only to the permeating Ca$^{2+}$ (for details see Ref. 7). In the resting state (−90 mV), the β subunit may stabilize an intercalation of the apo-CaM-LA complex in close vicinity of the ADSI, thus exposing CaM to the Ca$^{2+}$ influx at the intracellular opening of the pore. Mutation of ADSI, plasma-membrane anchoring of the α$_{1C}$ subunit C terminus, as well as uncoupling (by deletion or anchoring) of the α$_{1C}$ subunit N-tail in a β subunit-deficient channel, all deprive the channel of CDI, despite the presence of the C-terminal tail CaM-binding determinants LA and IQ. Inactivation of the channel (+20 mV) causes a voltage-gated rearrangement of the α$_{1C}$ C terminus (double arrow) that serves as a mobile carrier of the Ca$^{2+}$ signal for cAMP-response element-binding protein transcriptional activation (5).
12. Gurnett, C. A., De Waard, M., and Campbell, K. P. (1996) Neuron 16, 431–440
13. Felix, R., Gurnett, C. A., De Waard, M., and Campbell, K. P. (1997) J. Neurosci. 17, 6884–6891
14. Shirokov, R., Ferreira, G., Yi, J., and Rios, E. (1998) J. Gen. Physiol. 111, 807–823
15. Shibuya, H., Hara, M., Strobeck, M., Fukasawa, K., Schwartz, A., and Varadi, G. (1998) J. Biol. Chem. 273, 19348–19356
16. Yamaguchi, H., Hara, M., Strobeck, M., Fukasawa, K., Schwartz, A., and Varadi, G. (1998) J. Biol. Chem. 273, 19348–19356
17. Colecraft, H. M., Alseikhan, B., Takahashi, S. X., Chaudhuri, D., Mittman, S., Yegnasubramanian, V., Almaina, R. S., Johns, D. C., Marban, E., and Yue, D. T. (2002) J. Physiol. (Lond.) 541, 435–452
18. Hullin, R., Khan, I. F. Y., Wirtz, S., Mohacsi, P., Varadi, G., Schwartz, A., and Herzig, S. (2003) J. Biol. Chem. 278, 21623–21630
19. Takahashi, S. X., Mittman, S., and Colecraft, H. M. (2003) Biophys. J. 84, 3007–3021
20. Kobrinsky, E., Kepplinger, K. J. F., Yu, A., Harry, J. B., Kahr, H., Romanin, C., Abernethy, D. R., and Soldatov, N. M. (2004) J. Physiol. 541, 435–452
21. Bourou, A., Bouron, A., and Reuter, H. (1995) J. Biol. Chem. 270, 10540–10543
22. van der Wal, J., Habets, R., Varnai, P., Balla, T., and Jalink, K. (2001) J. Biol. Chem. 276, 15337–15344
23. Mori, M. X., Erickson, M. G., and Yue, D. T. (2004) Science 306, 491–495
24. Cuello, L. G., Cortes, D. M., and Perozo, E. (2004) Nature 419, 947–962
25. Howes, A. L., Arthur, J. F., Zhang, T., Miyamoto, S., Adams, J. W., Dorn, G. W., Woodcock, E. A., and Brown, J. H. (2003) J. Biol. Chem. 278, 40344–40351
26. Zhou, J., Olcese, R., Qin, N., Noceti, F., Birnbaumer, L., and Stefani, E. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2301–2305
27. Shirokov, R., Ferreira, G., Yi, J., and Rios, E. (1998) J. Gen. Physiol. 111, 807–823
28. Shibuya, H., Hara, M., Strobeck, M., Fukasawa, K., Schwartz, A., and Varadi, G. (1998) J. Biol. Chem. 273, 19348–19356
29. Birnbaumer, L., Qin, N., Olcese, R., Tareilus, E., Platano, D., Costantin, J., and Stefani, E. (1998) Bioenerg. Biomembr. 30, 357–375
30. Shibuya, H., Hara, M., Strobeck, M., Fukasawa, K., Schwartz, A., and Varadi, G. (1998) J. Biol. Chem. 273, 19348–19356
31. Colecraft, H. M., Alseikhan, B., Takahashi, S. X., Chaudhuri, D., Mittman, S., Yegnasubramanian, V., Almaina, R. S., Johns, D. C., Marban, E., and Yue, D. T. (2002) J. Physiol. (Lond.) 541, 435–452
32. Hullin, R., Khan, I. F. Y., Wirtz, S., Mohacsi, P., Varadi, G., Schwartz, A., and Herzig, S. (2003) J. Biol. Chem. 278, 21623–21630
33. Takahashi, S. X., Mittman, S., and Colecraft, H. M. (2003) Biophys. J. 84, 3007–3021
34. Kobrinsky, E., Kepplinger, K. J. F., Yu, A., Harry, J. B., Kahr, H., Romanin, C., Abernethy, D. R., and Soldatov, N. M. (2004) J. Physiol. 541, 435–452
35. Mori, M. X., Erickson, M. G., and Yue, D. T. (2004) Science 306, 491–495
36. Cuello, L. G., Cortes, D. M., and Perozo, E. (2004) Science 306, 491–495
37. Mori, M. X., Erickson, M. G., and Yue, D. T. (2004) Science 304, 432–435
38. Cuello, L. G., Cortes, D. M., and Perozo, E. (2004) Science 306, 491–495
39. Shibuya, H., Hara, M., Strobeck, M., Fukasawa, K., Schwartz, A., and Varadi, G. (1998) J. Biol. Chem. 273, 19348–19356
40. Bourou, A., Bouron, A., and Reuter, H. (1995) J. Biol. Chem. 270, 10540–10543
41. Mori, M. X., Erickson, M. G., and Yue, D. T. (2004) Science 304, 432–435
42. Cuello, L. G., Cortes, D. M., and Perozo, E. (2004) Science 306, 491–495
43. Shibuya, H., Hara, M., Strobeck, M., Fukasawa, K., Schwartz, A., and Varadi, G. (1998) J. Biol. Chem. 273, 19348–19356
44. Viard, P., Butcher, A. J., Halet, G., Davies, A., Nürnberg, B., Hebligh, F., and Dolphin, A. C. (2004) Nat. Neurosci. 7, 939–946