Modulation of L-type Ca\(^{2+}\) Channels by G\(\beta\)G and Calmodulin via Interactions with N and C Termini of \(\alpha_{1C}\)*

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Neuronal voltage-dependent Ca\(^{2+}\) channels of the N (\(\alpha_{1N}\)) and P/Q (\(\alpha_{1A}\)) type are inhibited by neurotransmitters that activate G\(_{\text{ia}}\) G proteins; a major part of the inhibition is voltage-dependent, relieved by depolarization, and results from a direct binding of G\(\beta\)G subunit of G proteins to the channel. Since cardiac and neuronal L-type (\(\alpha_{1L}\)) voltage-dependent Ca\(^{2+}\) channels are not modulated in this way, they are presumed to lack interaction with G\(\beta\)G. However, here we demonstrate that both G\(\beta\)G and calmodulin directly bind to cytosolic N and C termini of the \(\alpha_{1C}\) subunit. Coexpression of G\(\beta\)G reduces the current via the L-type channels. The inhibition depends on the presence of calmodulin, occurs at basal cellular levels of Ca\(^{2+}\), and is eliminated by EGTA. The N and C termini of \(\alpha_{1C}\) appear to serve as partially independent but interacting inhibitory gates. Deletion of the N terminus or of the distal half of the C terminus eliminates the inhibitory effect of G\(\beta\)G. Deletion of the N terminus profoundly impairs the Ca\(^{2+}\)/calmodulin-dependent inactivation. We propose that G\(\beta\)G and calmodulin regulate the L-type Ca\(^{2+}\) channel in a concerted manner via a molecular inhibitory scaffold formed by N and C termini of \(\alpha_{1C}\).

Voltage-dependent Ca\(^{2+}\) channels (VDCCs)\(^1\) are crucial for neuronal and muscular excitability (1). Mammalian VDCCs fall into several families distinguished by pharmacological and biophysical properties (L, N, P/Q, T, and R type) and the molecular identity of the main, pore–forming subunit, \(\alpha_1\) (2–4). The neuronal N- and P/Q-type channels, based on \(\alpha_{1B}\) and \(\alpha_{1A}\) respectively, are crucial for neurotransmitter release (3). L-type Ca\(^{2+}\) channels containing the “cardiac-type” \(\alpha_{1C}\) subunit regulate contraction of cardiac and smooth muscle, and excitability and gene expression in the brain (2, 5, 6). The \(\alpha_1\) subunits contain four homologous membrane domains numbered I–IV and 5 large intracellular segments: N terminus (NT), C terminus (CT), and linkers between the domains (often called loops L\(_1\), L\(_2\), and L\(_3\)). There is also a large number of short intracellular linkers between transmembrane segments within each domain.

Activation in all voltage-dependent channels is initiated by a voltage-driven shift in charged transmembrane elements (7). Nevertheless, the parts of the channel and the auxiliary subunits which are not exposed to the membrane electrical field may substantially modulate the gating (for reviews related to Ca\(^{2+}\) channels, see Ref. 3). In particular, VDCCs are strongly and specifically modulated by neurotransmitters acting via heterotrimeric G proteins, via actions on the cytosolic parts of the channel. Some of the modulations are mediated by G protein–triggered second messenger cascades, often via protein kinases A and C (PKA and PKC, respectively), others by a direct interaction with G protein subunits (1, 8–14). Both PKC and PKA alter VDCC gating parameters acting via cytosolic parts of \(\alpha_1\) or via the ancillary \(\beta\) subunit (15–19).

Neuronal VDCCs are usually inhibited by G protein-coupled receptors by voltage-independent (mediated by several second messenger pathways (20–23)) and voltage-dependent mechanisms. The latter is fast, membrane–delimited, mediated by G\(\beta\)G, and occurs in \(\alpha_{1A}\) (P/Q), \(\alpha_{1B}\) (N), and \(\alpha_{1E}\) (11–13). A hallmark of this modulation is relief of inhibition and acceleration of current activation by depolarization (voltage-dependent facilitation), which reflects a decrease in the affinity of the channel to G\(\beta\)G (24, 25). In \(\alpha_{1A}\), \(\alpha_{1B}\), and \(\alpha_{1E}\), G\(\beta\)G binds to L\(_1\) loop (15, 26, 27) and CT (27); NT is also important for the effect of G\(\beta\)G, although a direct interaction has not yet been established (28–30). The relative functional roles of the L\(_1\), CT, and NT-binding sites are still unclear (27, 31, 32).

Cytosolic parts of \(\alpha_1\) subunits are also involved in inactivation gating. A major part of inactivation of L-type VDCCs is triggered by the entry of Ca\(^{2+}\) (33). Calmodulin (CaM) has been recently identified as the Ca\(^{2+}\) sensor indispensable for the Ca\(^{2+}\)-dependent inactivation in L (\(\alpha_{1C}\)-) and P/Q (\(\alpha_{1A}\)-) type channels (34–37). A CaM-binding site has been identified in the CT of \(\alpha_{1C}\) and \(\alpha_{1A}\) Ca\(^{2+}\)-dependent CaM interaction with this domain has been found crucial not only for the Ca\(^{2+}\)-dependent inactivation, but also for an opposite effect of Ca\(^{2+}\), called Ca\(^{2+}\)-dependent facilitation (34–37).

Despite the outstanding role of cytosolic segments in channel modulation, our ideas of how these parts affect the gating of the VDCCs are vague. The L-type (\(\alpha_{1C}\)) channel is the best studied in this respect; but even here, only the roles of N and C termini have been examined. Removal of the distal half of the CT increases L-type channel currents and open probability by improving the coupling between gating charge (voltage sensor) movement and pore opening (38). It has been proposed that the CT acts as an inhibitory gate that conformationally restrains the opening of the channel (38, 39). Similarly to the CT, removal of the NT also enhances the open probability and the macroscopic currents in L-type channels, and a similar role (of an inhibitory gate) for the NT has been proposed (16, 17).

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\(^1\) The abbreviations used are: VDCC, voltage-dependent calcium channel; CaM, calmodulin; CT, C terminus; NT, N terminus; PKA, protein kinase A; PKC, protein kinase C; GST, glutathione S-transferase; aa, amino acid(s); Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)] ethylglycine; BAPTA, 1,2-bis(2-aminoethoxy)ethane-N,N,N’,N’-tetraacetic acid.

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39846
The L-type channel is not inhibited by neurotransmitters in a voltage-dependent manner (32, 40–42), and the $L_0$ loop of $\alpha_{1C}$ does not bind G$\beta$$\gamma$ (15, 26, 27). Voltage-dependent facilitation has been demonstrated in L-type VDCC, but it was PKA-rather than G-protein-dependent (Refs. 40 and 43; however, see Ref. 44). Therefore, $\alpha_{1C}$ has been assumed to lack any interaction with G$\beta$$\gamma$. $\alpha_{1C}$ even served in several studies as a donor of presumably G$\beta$$\gamma$-indifferent parts, to create chimeras with other $\alpha_1$ types, in the search for parts of $\alpha_1$ that determine the sensitivity to G$\beta$$\gamma$. However, here we demonstrate that G$\beta$$\gamma$ binds to NT and CT of $\alpha_{1C}$ and inhibits the L-type VDCC in a voltage-independent but calmodulin-dependent manner. We identify a novel CaM-binding site in the NT, which, like the previously identified CT-binding site, is an important determinant of the Ca$^{2+}$/CaM-dependent inactivation. We propose a model in which NT and CT of the L-type channel form a scaffold that plays a role of an inhibitory gate which integrates the regulatory effects of G$\beta$$\gamma$ and CaM.

EXPERIMENTAL PROCEDURES

cDNA Constructs and mRNA—The cDNAs of the G protein subunits (bovine G$\beta_2$, human G$\beta_3$, and bovine G$\gamma_2$, provided by M. Simon, Caltech) were either amplified by polymerase chain reaction to create EcoRI sites at the 5′ and 3′ ends (G$\beta_2$ and G$\gamma_2$) or excised with EcoRI from the original vectors (all the others) and subcloned into the EcoRI site of the pGEMHE (G$\beta_3$, G$\beta_2$, and G$\gamma_2$) and pGEMHI (G$\beta_2$ and G$\beta_3$) vectors (45, 46). The cDNAs of CaM and CaM$_{D_{1700}}$ (47) were provided by J. P. Adelman. cDNAs and RNAs of rabbit Ca$^{2+}$ channels subunits $\beta_A$ and $\alpha/\delta$ were as described (48). The rabbit heart $\alpha_{1C}$ cDNA (49) and all its mutants used here were subcloned into Sal/HindIII sites of the pGEM-SB vector (16). The N-terminal deletion mutant of $\alpha_{1C}$, $\Delta_{N_{1-139}}$, was prepared as described (16). To create the $\alpha_{1C}$ C-terminal truncation mutant $\Delta_{C_{700-end}}$ polymerase chain reaction amplification of a C-terminal part of $\alpha_{1C}$ was performed to create a stop codon after nucleotide 5274 (numbering by Ref. 49) followed by a HindIII site. The truncated cDNA was subcloned back into GEM-SB vector. The 3′UTR was constructed by cutting and ligating the appropriate parts of $\Delta_{N_{1-139}}$ and $\Delta_{C_{700-end}}$ mutants. The RNAs were prepared using a standard procedure (50).

cDNAs designed to create glutathione S-transferase (GST) fusion proteins were constructed using polymerase chain reaction strategy, with primers containing the desired restriction sites and linked in-frame to GST, as described (16). The cDNA constructs encode the GST fusion proteins of the following segments of $\alpha_{1C}$: whole N terminus (N$_1$–1354); CT and three C-terminal cDNA fragments (C, aa 1505–2171; C$_0$, aa 1505–1846; C$_1$, aa 1664–1845; and C$_2$, aa 1841–2171); L$_1$, aa 438–550; L$_2$, aa 783–930; and L$_3$, aa 1196–1249.

Oocytes and Electrophysiology—Xenopus laevis frogs were maintained as described (50). Oocytes were injected with equal amounts (by weight) of the mRNAs of $\alpha_{1C}$, or its mutants with or without $\beta_A$, and incubated for 3–5 days at 20–22 °C in NDE96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 2.5 mM Na pyruvate, 50 $\mu$g/ml gentamicine, 5 mM HEPES, pH 7.5). Whole cell currents were recorded using the Gene Clamp 500 amplifier (Axon Instruments, Foster City, CA) using the two-electrode voltage clamp technique in a solution containing 40 mM Ba(OH)$_2$ or 40 mM Ca(OH)$_2$, 50 mM NaOH, 2 mM KOH, and 5 mM HEPES, titrated to pH 7.5 with methanesulfonic acid. In some cases, a solution with 2 mM Ba$^{2+}$ was used (2 mM Ba(OH)$_2$, 96 mM NaOH, 2 mM KOH, 5 mM HEPES, pH titrated to 7.5 with methanesulfonic acid). Stimulation, data acquisition, and analysis were performed using pCLAMP software (Axon Instruments). Ba$^{2+}$ currents were measured by a 200 or 400 ms step to 20 mV from a holding potential of ~80 mV.

Interaction between GST Fusion Proteins and in vitro Synthesized G$\beta_3$ and Calmodulin—The procedures were essentially as described (16). In brief, [35S]Met/Cys-labeled G$\beta_3$, G$\gamma_2$, CaM, and CaM$_{D_{1700}}$ were translated on the template of in vitro synthesized RNAs using a rabbit reticulocyte translation kit (Promega). The fusion proteins were synthesized from E. coli using the Amersham Pharmacia Biotech kit. Purified GST fusion proteins (5–10 $\mu$g) or purified GST (10 $\mu$g) were incubated with 5 $\mu$L of the lysate, containing the 35S-labeled proteins in 500 $\mu$L of phosphate-buffered saline with 0.05% Tween 20, for 2 h at room temperature, with gentle rocking. In some experiments the incubation was done in the presence of 1 mM CaCl$_2$ or 5 mM EGTA. In the experiments shown in Fig. 5C, the incubation was done in the same buffer but with varying concentrations of free Ca$^{2+}$, in the presence of 2 mM EGTA: free Ca$^{2+}$ concentration was calculated using the MAXC program. Then 30 $\mu$L of glutathione-Sepharose beads (Amersham Pharmacia Biotech) were added, and the mixture was incubated for 30 min at 4 °C and washed four times in 1 mL of the same buffer. Following washing, GST fusion proteins were eluted with 30 $\mu$L of 20 mM reduced glutathione in elution buffer (120 mM NaCl, 100 mM Tris-HCl, pH 8). CaM was analyzed on 15% G$\beta_3$, on 12% SDS-polyacrylamide gels. G$\gamma_2$ was analyzed on Tricine ready-made gels (Bio-Rad). The labeled products were identified and quantified by autoradiography using a PhosphorImager (Molecular Dynamics) as described (51). Phosphorylation of the N-terminal GST fusion protein was performed as described (17).

Immunocytochemistry of the Expressed $\alpha_{1C}$ in Xenopus Oocytes—This was performed essentially as described (51, 52). Oocytes were injected with mRNAs and incubated in NDE solution containing 0.5 mM $[35S]$methionine/cysteine (Amersham Pharmacia Biotech) for 4 days at 22 °C. Plasma membranes were separated manually (51) from the rest of the oocyte (designated as internal fraction). 10–20 membranes and 3–5 internal fractions, or 5 whole oocytes, were homogenized, proteins were solubilized, immunoprecipitated as described (16, 52), and electrohoresed on 6 or 3–8% polyacrylamide-SDS gels. Card-1 antibody was kindly provided by M. M. Hosey (Northwestern University, Chicago) (53).

RESULTS

G$\beta_3$ Binds to Intracellular Segments of $\alpha_{1C}$—Direct interaction between intracellular segments of $\alpha_{1C}$ with G$\beta_3$ was studied in vitro using fragments of $\alpha_{1C}$ fused to GST, covering all the large intracellular segments of $\alpha_{1C}$ (Fig. 1A). They included the whole NT (N$_1$–154), the full-length CT (C), three subdivisions of the C terminus (C$_0$, C$_1$, and C$_2$, as shown in Fig. 1A, inset), and three interdomain linker loops (L$_1$, L$_2$, and L$_3$). The binding of G$\beta_3$ to NT, CT, or its parts, and L$_3$ has not been examined in the past. The GST fusion proteins were immobilized on glutathione-agarose beads and assayed for interaction with in vitro translated, 35S-labeled G$\beta_2$,$\gamma_2$ subunits. Unexpectedly, NT and CT bound G$\beta_2$,$\gamma_2$ whereas GST alone and loops L$_1$, L$_2$, and L$_3$ did not bind G$\beta_3$ (Fig. 1B, upper panel). The results of all experiments were quantitated by normalizing the 35S-G$\beta_3$ signal obtained from each GST fusion protein to that of the NT obtained in the same experiment (Fig. 1B, lower panel). The CT exhibited the strongest interaction with G$\beta_3$, mainly localized to its proximal half (C$_0$, aa residues 1505–1846). The distal half of the CT, C$_1$, showed a weak but reproducible G$\beta_3$ binding. Since there is no binding in the middle of CT (C$_0$), it is plausible that G$\beta_3$ binds to two separate sites in the CT, roughly in its first quarter (from the beginning of C$_0$ to the beginning of C$_1$ between aa 1505 and 1664) and the last half. NT showed intermediate G$\beta_3$ binding. Fig. 1C shows that the binding of G$\beta_3$ to NT and CT was Ca$^{2+}$-independent: it was identical in the presence of 1 mM Ca$^{2+}$ or 5 mM EGTA, or with no additions (control). The binding of G$\beta_3$ to NT (which is a PKC substrate: see Ref. 17) was not affected by phosphorylation by PKC or by the presence of the Ca$^{2+}$ channel $\beta_2$-$\alpha$ subunit (data not shown).

Of the 5 known G$\beta_3$ proteins (55), the highly homologous G$\beta_3$ through G$\beta_6$ often show considerable selectivity in modulating the voltage-dependent Ca$^{2+}$ channels (Refs. 56–58; but see Ref. 59). However, in $\alpha_{1C}$ all four $\beta$ subunits (presented, in all cases, with G$\gamma_2$) bound well to NT and CT (Fig. 1D). G$\delta$ could...
bind to NT of α1C without the Gγ, while Gγ2 did not bind (Fig. 1E), as also has been shown for L1 of α1B (58).

Coexpression of Gβγ Attenuates the L-type Ca$^{2+}$ Channel Currents—Although L-type Ca$^{2+}$ currents in neurons are down-regulated by activation of G$\alpha_{lo}$-coupled neurotransmitter receptors (see “Discussion”), such modulations could not be reproduced in two expression systems, oocytes and HEK cells (31, 32, 40–42). It seemed the Gβγ, when released from G proteins after activation of the relevant neurotransmitters by agonists, did not directly regulate the L-type channel. This leaves open the question of the functional consequences of the interaction between α1C and Gβγ revealed by experiments of Fig. 1. To address this problem, we used coexpression methodology. L-type channels were expressed in Xenopus oocytes in full subunit composition (α1C, αδ and β2A), or without the β subunit (α1Cαδβ combination). Currents were measured using the two-electrode voltage clamp technique. At standard levels of expression of the channel used here (1 ng of RNA/oocyte for α1Cαδβ, 2.5 ng/oocyte for α1Cαδ), the average $I_{Ba}$ was 1064 ± 47 nA (n = 58 oocytes, N = 8 batches) and 324 ± 15 nA (n = 149, N = 19), respectively.

In accord with previous reports, activation by acetylcholine of a coexpressed muscarinic m2 receptor (which couples to G$\alpha_{lo}$ proteins) did not cause any consistent modulation of $I_{Ba}$ (data not shown). However, upon additional coexpression of G$\alpha_{lo}$, which is indispensable for muscarinic inhibition of L-type Ca$^{2+}$ channels in pituitary neurons (60), acetylcholine caused a substantial inhibition of $I_{Ba}$ in 2 out of 5 oocyte batches tested (data not shown). The inconsistency of modulation suggests that additional unidentified proteins (lacking in some oocyte batches) are important for the effect of acetylcholine.

In contrast, coexpression of Gβγ reproducibly decreased $I_{Ba}$ by ~40% in all oocyte batches tested (Fig. 2, A and C). In each batch, currents were normalized to the average $I_{Ba}$ in control group; this allowed the comparison and quantitation of effects of Gβγ coexpression in many batches. At standard levels of channel expression, the current decrease caused by the coexpression of Gβγ in the full subunit combination was 39.4 ± 2.7% (n = 58 oocytes from 8 batches), and in α1Cαδ combination the decrease was 38.6 ± 2% (n = 149 oocytes from 19 batches; Fig. 2C). The inhibitory effect of coexpressed Gβγ on $I_{Ba}$ was suppressed by coexpression of Gβγ scavengers: free G$\alpha_{1}$, and the myristoylated C-terminal fragment of GIRK1+ channel, GIRK183–501src (61) (data not shown). The inhibition by Gβγ was clearly dose-dependent; a maximal inhibition was observed already at 0.1–1 ng/oocyte Gβ RNA (0.1–0.2 ng/oocyte Gγ RNA), and half-maximal effective doses were 0.17 and 0.07 ng/oocyte Gβ RNA for the full and α1Cαδβ subunit combinations, respectively (Fig. 2B). In all following experiments, Gβ and Gγ RNAs were always injected at supramaximal doses, 5 and 1 ng/oocyte, respectively.

We noticed that the inhibitory effect of Gβγ coexpression became smaller when more channels were expressed. This was investigated with α1Cαδβ channels expressed in increasing amounts by injecting 1.25, 2.5, or 5 ng/oocyte of each subunits RNA (Fig. 2C). At the highest concentration of channel RNA, the inhibition was lost and even replaced by a small but statistically significant 17 ± 5% enhancement (Fig. 2C, right panel). The same trend was observed in the channels of full subunit combination (Fig. 2C, left panel). Gβγ containing any of the four Gβ subunits tested in Fig. 1, Gβ1 through Gβ4, inhibited $I_{Ba}$ approximately to the same extent as Gβ1 with the standard RNA concentrations (Fig. 2D).

To exclude the possibility of a Gβγ effect on the level of Ca$^{2+}$ channel expression, in two separate oocyte batches the RNA of Gβ1γ2 was injected 4 days after the injection of RNAs of α1 and αδβ, and $I_{Ba}$ was measured 3 days later. $I_{Ba}$ reached a steady level 3–4 days after RNA expression; data not shown.) In these experiments, the decrease in $I_{Ba}$ was 35.5 ± 3.7% (n = 16, N = 2).
The effect of coexpression of \( G_{\beta y} \) on the total level of expression of \( \alpha_{1C} \) protein was further studied by quantitative immunoprecipitation of \(^{35}\)S-labeled \( \alpha_{1C} \) from \textit{in vitro} metabolically labeled oocytes. \( \alpha_{1C} \) was immunoprecipitated from manually separated plasma membranes and from the rest of the oocytes ("internal fraction") as described (51). Previously, we used 5 ng of RNA/oocyte to visualize \( \alpha_{1C} \) in plasma membranes (16). This time, to assure that these biochemical measurements are compatible with the electrophysiological ones described above, we used the standard RNA doses (2.5 ng/oocyte; \( \alpha_{1C}d \alpha_{3}, \delta \) subunit combination). Despite the low level of \( \alpha_{1C} \) protein expression in the plasma membrane obtained under these conditions, it was clear that the amount of \( \alpha_{1C} \) in the plasma membrane was rather increased by coexpression of \( G_{\beta y} \) (Fig. 2E, \textit{left panel}). Similarly, in the internal fraction, the amount of \( \alpha_{1C} \) protein observed in the internal fraction containing the cytosol and the intracellular membranes (Fig. 2E, \textit{right panel}).

Roles of \( N \) and \( C \) Termini in Gating and in Mediating the \( G_{\beta y} \) Effects — To examine the functional impact of interaction with \( G_{\beta y} \) with \( N \) and \( C \) termini, we expressed three truncated constructs of \( \alpha_{1C} \): \( \Delta C_{1700-\text{end}} \) ("\( \Delta C \)) which lacks the distal part of the \( \alpha_{1C} \) C-terminus, \( \Delta N \) ("\( \Delta N \)) which lacks most of the \( \alpha_{1C} \) N-terminus, and the double deletion mutant \( \Delta N \Delta C \), missing both pieces. Since it has been shown previously that neither NT nor CT deletions significantly alter the amount of \( \alpha_{1C} \) in the plasma membrane (16, 38), we injected the standard amounts of channel RNA to obtain similar levels of channel expression. Wild-type \( \alpha_{1C} \) and truncated \( \alpha_{1C} \) were coexpressed with \( \alpha_{2\delta} \) with or without \( G_{\beta y} \). Peak \( I_{Ba} \) was increased 10-, 22-, and 80-fold by the deletion of the NT, CT, and both termini, respectively (Fig. 3A). The current-voltage (I-V) relationship was not significantly affected by each individual deletion, but I-V of \( \Delta N \Delta C \) was shifted to the left (Fig. 3B). Fig. 3C shows that all deletions resulted in abolition of the \( G_{\beta y} \)-induced decrease in \( I_{Ba} \) (which was 26.1 ± 3.7% in these three experiments). In all cases, the inhibition was lost and, in \( \Delta C_{1700-\text{end}} \) and \( \Delta N \Delta C \), there was even a small enhancement of the current. Thus, both \( N \) and \( C \) termini are indispensable for the \( G_{\beta y} \)-induced inhibition.

The Inhibitory Effect of \( G_{\beta y} \) is \( Ca^{2+} \)- and Calmodulin-dependent — Although Ba\(^{2+} \) was used as the charge carrier, we were surprised to find that, in oocytes injected shortly before the experiment with a high-affinity \( Ca^{2+} \) chelator, EGTA, the inhibition by coexpressed \( G_{\beta y} \) was lost (Fig. 4A),
suggestions that the Gβγ-induced inhibition requires the presence of a certain basal level of Ca2+. Since Gβγ binding to CT and NT was Ca2+-independent (Fig. 1), it was plausible that a Ca2+-binding protein such as CaM was involved in the Gβγ-induced inhibition. To test this idea, we coexpressed the L-type channel with either the wild-type calmodulin, CaM WT, or its dominant negative mutant, CaM1234 (47). Interestingly, expression of CaM itself increased Ia,b. At the standard level of channel expression (2.5 ng of RNA/oocyte; α1,α2,β channels), the increase was not significant (22.6 ± 12.7%; n = 20, N = 3; p < 0.12). However, at the high level of channel expression (5 ng/oocyte) there was a large, highly significant increase in Ia,b (63.6 ± 15%; n = 20, N = 3; p < 0.001). This is in line with the notion that, at lower channel levels, there is enough endogenous CaM; therefore, the effect of expressed CaM becomes evident only at high levels of expressed channels. Similar results were obtained when CaM RNA was injected 2–3 days after the channels RNA (data not shown). In contrast, CaMWT reduced Ia,b by 31 ± 10% (n = 13, N = 2, p < 0.05) at the standard channel concentration.

As shown in Fig. 4B, at standard levels of channel expression, CaMWT did not alter the effect of Gβγ, while CaM1234 significantly attenuated the Gβγ-induced inhibition. These results support the assumption that CaM may participate in Gβγ-dependent inhibition. It was therefore likely that CaM may be the endogenous factor “missing” for the inhibitory effect of Gβγ when high doses of channel are expressed. To examine this assumption, we coinjected either standard (2.5 ng/oocyte) or high (5 ng/oocyte) doses of RNA of α1 and α2,β subunits, with RNAs of Gβγ and CaMWT. As in the previous experiments, we observed the inhibitory effect of Gβγ on Ia,b with the standard RNA concentration (Fig. 4C, left panel). The opposite effect of Gβγ when the channel was expressed at a higher level (Fig. 4C, right panel). Coexpression of CaMWT together with Gβγ in the conditions of high level of expression of the channel resulted in a partial restoration of the inhibitory effect of Gβγ (Fig. 4C, right panel). None of the effects of CaM coexpression could be attributed to a change in the level of α1C in the oocytes,
BAPTA was compared using the protocol of Peterson (vitro). These interactions were Ca\textsuperscript{2+} dependent inactivation was attenuated by the removal of the NT. G\textsubscript{bg} affects the Ca\textsuperscript{2+}-dependent inactivation remained unaffected by coexpression of G\textsubscript{bg}, either in the absence or presence of CaM\textsubscript{1234}. The inactivation of Ba\textsuperscript{2+} currents was not affected by either G\textsubscript{bg} or CaM\textsubscript{1234}(Fig. 6). Thus, coexpression G\textsubscript{bg} did not affect the inactivation process.

**DISCUSSION**

This work urges to reconsider the view that L-type Ca\textsuperscript{2+} channels lack interaction with G\textsubscript{bg} and also provides new insights into the mechanisms of L-type channel gating and its modulation by calmodulin. We demonstrate that NT and CT of \alpha\textsubscript{1C} are partially independent, interacting inhibitory gates. G\textsubscript{bg} directly binds to NT and CT. Coexpression of G\textsubscript{bg} reduces the L-type Ca\textsuperscript{2+} channel currents; this inhibition requires the presence of CaM. CaM binds to CT and to a novel site in NT, which plays an important role in Ca\textsuperscript{2+}/CaM-dependent inactivation.

**Interaction of the \alpha\textsubscript{1C} Subunit of the L-Type Channel with G\textsubscript{bg} Affects the Channels Function**—Using GST fusion proteins of cytosolic segments of \alpha\textsubscript{1C}, we demonstrate that G\textsubscript{bg} binds to the NT and to two separate sites in the CT of \alpha\textsubscript{1C}. The strongest interaction is in the first half of the CT and is probably confined to the first quarter of the CT. The NT shows weaker but also substantial G\textsubscript{bg} binding. In agreement with previous reports (15, 26, 27), the L\textsubscript{1} loop of \alpha\textsubscript{1C} does not bind G\textsubscript{bg}, and no substantial binding is seen in other intersegment linker loops. Some isoforms of neuronal \alpha\textsubscript{1C} have a shorter N terminus than the cardiac-type \alpha\textsubscript{1C} used here (62). However, \alpha\textsubscript{1C} with a full-length N terminus is also abundant in the brain, at least in rat
The role of CaM in regulation of L-type Ca\(^{2+}\) channel activity is well known. The presence of CaM has been shown to enhance the activity of the channel by binding to the C-term of the channel, facilitating its interaction with other proteins, and modulating the channel's activity. CaM also plays a role in the regulation of channel activity in response to various stimuli, such as neurotransmitters or other signaling molecules. The interaction between CaM and the channel is complex and involves multiple binding sites and mechanisms. The role of CaM in regulating the activity of L-type Ca\(^{2+}\) channels is crucial for maintaining proper cellular function and is essential for the proper functioning of many physiological processes, including excitation-contraction coupling in cardiac muscle.
L-type Ca\textsuperscript{2+} Channel Regulation by G\textsubscript{bg} and Calmodulin

not interact with GST fusion proteins of either NT or CT at basal Ca\textsuperscript{2+} levels (Fig. 5), higher affinity may be envisioned within a hypothetical binding site formed jointly by NT and CT (see below). Thus, theoretically, basal Ca\textsuperscript{2+} levels may suffice to support CaM-\(\alpha_{1C}\) interaction and thus G\textsubscript{bg} inhibition, but this point requires further clarification. Second, CaM is an abundant cellular protein. It seems surprising that there is not enough for the modulation of Ca\textsuperscript{2+} channels when they are expressed at relatively high levels. However, most of the cellular CaM may not be free but pre-assembled with its other effectors.

Role of N and C Termini in L-type Ca\textsuperscript{2+} Channel Gating: an NT/CT Scaffold Model—In L-type Ca\textsuperscript{2+} channels, NT and CT appear to act as inhibitory gates that attenuate the channels activation by depolarization; their removal increases the ionic currents without an increase in expression levels (16, 38, 39). Joint removal of both NT and CT1700-end (in the \(\alpha_{1C}\) mutant), performed here for the first time, revealed an even greater increase in the macroscopic ionic currents than the removal of each terminus separately. Peak 1\textsubscript{Ba} was increased ~10-fold by the deletion of the NT, ~22-fold by the deletion of the CT1700-end, and ~80-fold by the deletion of both (Fig. 3). Thus, each inhibitory gate (NT and CT) has an inhibitory effect of its own, and neither one is permissive for the other, otherwise the removal of either one would already have caused a maximal effect.

On the other hand, the maximal effect of the joint removal of two independent gates would be a multiple of the individual effects, i.e. a 220-fold increase in 1\textsubscript{Ba} (the open probability of a single \(\alpha_{1C}\) channel is below 0.001 in (2), and such a change is theoretically possible). Furthermore, the current-voltage curve was not significantly affected by either CT or NT deletion (16, 38, 39), but was shifted to the left by deletion of both termini (Fig. 3), suggesting their synergistic effect on activation gating. Finally, both NT and CT were indispensable for the G\textsubscript{bg}-induced inhibition: removal of either terminus eliminated the inhibition. Therefore, we conclude that CT and NT are interacting gates. Importantly, the major G\textsubscript{bg}-binding site of the CT is located before aa 1700. The fact that the deletion of the CT beyond aa 1700 fully eliminates the G\textsubscript{bg} effect implies that it is the removal of the gating element, rather than of the G\textsubscript{bg}-binding site, which causes this effect. (Alternatively, it may suggest a great functional importance for the weak G\textsubscript{bg}-binding site found in the second half of CT.)

Based on these considerations, we propose that C and N termini act as partially independent but interacting gates that form a molecular scaffold at the cytoplasmic side of the channel, which deters channel opening, probably by an allosteric mechanism. Its conformation changes as a function of the presence of Ca\textsuperscript{2+} and of the extent of interaction between G\textsubscript{bg} and CaM; other cytosolic parts of \(\alpha_{1C}\) and the Ca\textsuperscript{2+}-channel \(\beta\) subunit may contribute to scaffolds formation. We propose that factors that “tighten” the interaction between NT and CT strengthen the inhibitory control exerted by the scaffold. Fig. 7 presents a simplified scheme of this model and of the way G\textsubscript{bg} and CaM regulate gating. The scaffold harbors at least two binding sites for each CaM and G\textsubscript{bg}, in each of the termini. G\textsubscript{bg} binds to the scaffold in the absence of elevated Ca\textsuperscript{2+} and reduces the current by strengthening the interaction between CT and NT and/or by opposing a “basal” facilitating effect of CaM. Influx of Ca\textsuperscript{2+} via the open channel is followed by Ca\textsubscript{2+}-binding to CaM which, in turn, improves the interaction of CaM with its binding sites and further tightens the scaffold, causing more inhibition (which is apparent as Ca\textsuperscript{2+}-dependent inactivation). The latter effect is G\textsubscript{bg}-independent.

This model actually presents a further elaboration of that proposed by Peterson et al. (37) to explain Ca\textsuperscript{2+}-independent inactivation. It has been envisaged that CaM, which in the absence of Ca\textsuperscript{2+} must be tethered to a site distinct from the C-terminal IQ domain, binds to the latter following Ca\textsuperscript{2+} influx and binding. Together with the yet undefined tethering element, the CT has been proposed to form a scaffold which, after binding of Ca\textsuperscript{2+}/CaM, induces inactivation. The necessity for a CaM tethering element that should bind CaM in the absence of Ca\textsuperscript{2+} was also stressed by Qin et al. (34) and Zuhlke et al. (36). However, although we have tested the binding of CaM to all large cytosolic parts of \(\alpha_{1C}\), no Ca\textsuperscript{2+}-independent binding sites could be found. Furthermore, the CaM\textsubscript{1234} mutant, which is supposed to act by replacing the endogenous WT CaM at the tethering site, did not bind to any of our GST fusion proteins either in the absence or presence of Ca\textsuperscript{2+}. Possibly, the three-dimensional structure of the NC/terminal scaffold is such that the NT and CT CaM-binding sites actually form one, high-affinity site which may anchor CaM (or CaM\textsubscript{1234}) in the absence of elevated Ca\textsuperscript{2+} (alternatively, the small linkers within the homology domains may contribute to the anchoring site). A high-affinity site for G\textsubscript{bg} may also be jointly formed by the three sites (NT, proximal CT, distal CT) identified here, eventually providing for a 1:1 stoichiometry for G\textsubscript{bg} binding (as appears to be the case with \(\alpha_{1B1}\); see Ref. 25).

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Fig. 7. Schematic representation of the NT/CT scaffold model. A cartoon of channel states is shown at the top; corresponding Ba\textsuperscript{2+} or Ca\textsuperscript{2+} currents are drawn at the bottom. In the resting state, at basal cellular Ca\textsuperscript{2+} levels, NT and CT are loosely folded; CaM may be anchored with only a fraction of its Ca\textsuperscript{2+}-binding sites occupied by Ca\textsuperscript{2+}; depolarization causes large, poorly inactivating currents in a high-Ba\textsuperscript{2+}, Ca\textsuperscript{2+}-free solution (left). Binding of G\textsubscript{bg} causes tightening of the scaffold and a decrease in the current (middle). A switch to a Ca\textsuperscript{2+}-containing solution and the entry of Ca\textsuperscript{2+} causes a forceful tightening of the scaffold and inactivation, due to strong binding of CaM to its NT- and CT-binding sites; this process is largely unaffected by G\textsubscript{bg}.
Modulation of L-type Ca\textsuperscript{2+} Channels by G\textsubscript{\beta\gamma} and Calmodulin via Interactions with N and C Termini of \( \alpha_{1C} \)

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