Differential Interactions of the C terminus and the Cytoplasmic I-II Loop of Neuronal Ca\(^{2+}\) Channels with G-protein \(\alpha\) and \(\beta\gamma\) Subunits

I. MOLECULAR DETERMINATION*

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A family of membrane-associated guanine nucleotide-binding regulatory proteins (G-proteins) is essential for mediating signal transduction between cell-surface receptors and intracellular effectors such as adenylate cyclase, phospholipase C, phospholipase A\(_2\), and ion channels (1–4). G-proteins are composed of three subunits termed \(\alpha\), \(\beta\), and \(\gamma\). The \(\alpha\) subunit (Go) contains a binding site for guanine nucleotides and possesses GTPase activity. Upon receptor stimulation, heterotrimeric G-proteins disassociate into an \(\alpha\)-GTP complex and \(\beta\gamma\) dimer.

Interactions of G-protein \(\alpha\) (Go) and \(\beta\gamma\) subunits (G\(\beta\gamma\)) with N- (\(\alpha_{1B}\)) and P/Q-type (\(\alpha_{1C}\)) Ca\(^{2+}\) channels were investigated using the Xenopus oocyte expression system. G\(\alpha\) was found to inhibit both N- and P/Q-type channels by receptor agonists, whereas G\(\beta\gamma\) was responsible for prepulse facilitation of N-type channels. L-type channels (\(\alpha_{1C}\)) were not regulated by Go or G\(\beta\gamma\).

For N-type, prepulse facilitation mediated via G\(\beta\gamma\) was impaired when the cytoplasmic I-II loop (loop 1) was deleted or replaced with the \(\alpha_{1C}\) loop 1. Go-mediated inhibitions were also impaired by substitution of the \(\alpha_{1C}\) loop 1, but only when the C terminus was deleted. For P/Q-type, by contrast, deletion of the C terminus alone diminished Go-mediated inhibition. Moreover, a chimera of L-type with the \(\alpha_{1n}\) loop 1 gained G\(\beta\gamma\)-dependent facilitation, whereas an L-type chimera with the N- or P/Q-type C terminus gained Go-mediated inhibition. These findings provide evidence that loop 1 of N-type channels is a regulatory site for G\(\beta\gamma\) and the C termini of P/Q- and N-types for Go.

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Most systems, a GTP-bound Go activates or inhibits an effector system, and the functional half-life is determined by the intrinsic GTPase activity of Go. Recently, it has been shown that the \(\beta\gamma\) dimer (G\(\beta\gamma\)) is significantly important in signal transduction as well (3).

High voltage-activated (HVA) Ca\(^{2+}\) channels are negatively regulated by G-proteins in a membrane-delimited manner (2, 4). This response is primarily mediated by pertussis toxin-sensitive G-proteins (G\(\alpha\)) in which G\(\alpha\) has been shown to inhibit current from HVA Ca\(^{2+}\) channels (5–7). Additionally, it has been shown that G\(\beta\gamma\) also transduces an inhibitory signal to HVA Ca\(^{2+}\) channels (8, 9). It remains to be determined, however, which subunit arm of the G-protein complex preferentially interacts with N- and P/Q-types of HVA Ca\(^{2+}\) channels. Recently, it has been determined that the intracellular loop joining motif I and II (referred to as “loop 1” in the present study) is an interaction site on neuronal HVA Ca\(^{2+}\) channels for G\(\beta\gamma\) (10–13). Nevertheless, mapping of region(s) on HVA Ca\(^{2+}\) channels responsible for interactions with Go and/or G\(\beta\gamma\) is still very incomplete (14).

To address these issues at the molecular level, we have functionally expressed \(\alpha_{1A}\), \(\alpha_{1B}\), and \(\alpha_{1C}\) of HVA Ca\(^{2+}\) channels in Xenopus oocytes. These subunits were derived from rabbit brain N-type, P/Q-type, and cardiac L-type Ca\(^{2+}\) channels, respectively. In addition, we have co-expressed \(\delta\) opioid receptor (DOR) together with Go or G\(\beta\gamma\) as we did in determining a region of the muscarinic-gated K\(^+\) channel critical for activation by G\(\beta\gamma\) with the presumption that co-expression with Go or G\(\beta\gamma\) determines which kind of modulation takes place (15). In this paper, interactions of Go and G\(\beta\gamma\) with Ca\(^{2+}\) channels were characterized using mutant and chimeric N- (\(\alpha_{1N}\)) and P/Q-type (\(\alpha_{1P}\)) Ca\(^{2+}\) channels. The results, together with evidence for a direct binding provided by the companion paper (16), define the interaction sites of Ca\(^{2+}\) channels for Go and G\(\beta\gamma\).

EXPERIMENTAL PROCEDURES

In Vitro Transcription

The 1.4-kb ApaI/ApaI and 6.8-kb HindIII/ HindIII fragment containing the entire coding regions of DOR (17) and the Ca\(^{2+}\) channel \(\alpha_{1C}\) subunit (18) were inserted into the HindIII site of the pSpA2 vector (19), to yield pSpDOR and pSpCDR, respectively. The plasmid pSpCDR was digested with XhoI, blunted with T4 DNA polymerase, and ligated with a SalI linker to yield pSpCDRS. The \(\alpha_{1C}\) subunit cDNA was kindly donated by Drs. Atsushi Mikami and Tetsuo Tanimasa. The pSpA1, pSpA2, pSpT7, pSp65, and pSp64 recombinant plasmids carrying the entire protein-coding sequences of G\(\alpha\), G\(\beta\), G\(\gamma\), and Ca\(^{2+}\) channel...
The plasmid pSPB3 coding for amino acid residues 61–2177 of the α1B subunit was substituted for amino acid residues 332–668 of the a subunit, the plasmid pSP3DND1. In this plasmid, the codon AGG and CCC for Arg-1911 of the α1B subunit was substituted for amino acid residues of 1658–2171 of the α1C subunit with a deletion of the C-terminal amino acid residues 1925–2573.

Subcloning and mutagenesis procedures were verified by restriction enzyme analysis and DNA sequencing.

**Functional Expression of Wild-type, Mutant, and Chimeric Ca2⁺ Channels in Xenopus Oocytes**

After removal of the follicular cell layer (15), Xenopus oocytes were injected either with 0.3 μg/μl α1 (α1m, α1n, α1c, or chimeric α1) cRNA, or 0.5 μg/μl βα or chimeric βα cRNA, or 0.05 μg/μl OR cRNA, or 0.05 μg/μl Gαi cRNA, or 0.05 μg/μl Gβγ cRNA, or 0.025 μg/μl Gγ cRNA, unless otherwise specified. The average volume of injection was ~50 nl per oocyte. The injected oocytes were incubated for 3–5 days and then subjected to electrophysiological measurements at 21 ± 2 °C.

In order to mask the effect of endogenous Go (16), a deoxyoligonucleotide 20-mer (AGO) of the following sequence was used in anti-sense experiments, CATGACTGCTGGGGGGGG. The AGO anti-sense oligonucleotide is complementary to nucleotides (17–18 of) the Xenopus Gαa mRNA (23). The endogenous Xenopus Gαa nucleotide sequence shows 40% identity with the corresponding nucleotide sequence of Gαa cRNA injected. This antisense oligonucleotide (0.1 μg/μl, 50 n) was injected 12–16 h prior to electrophysiological measurements.

**Construction of Mutant and Chimeric Ca2⁺ Channels**

These plasmids were constructed by site-directed mutagenesis of the plasmid pSPB3. The resulting plasmids were verified by restriction enzyme analysis and DNA sequencing.

**Interaction of Ca2⁺ Channels with Ga and Gβγ**

α1m, α1n, and βα subunits were described previously (15, 18–21). Nucleotide sequence analyses revealed that the deduced amino acid sequence of Gαa was the same as that reported (22) except that Ser-16, Asp-64, Ser-165, Gln-261, and Pro-282 were determined as Thr (ACG), Glu (GAA), Thr (ACC), Asp (GAC), and Ser (CTA), respectively, in our clone C (5).

cRNAs specific for α1m, α1n, α1c, and βα subunits of the Ca2⁺ channel, 17 kinds of mutants and chimeric α1 subunits (see below), DOR, Gαα, Gβγ, and Gγ, were synthesized in vitro using a MEGAscript SP6 kit (Ambion).

The resulting plasmid and the plasmid pSPCDTB3 were digested with SacI and blunted with T4 DNA polymerase, and cleaved with NotI, PvuII, XmnI, and/or PstI. The 1.1-kb NotI/SacI and 510-bp PvuII/PstI fragments, the 1.2-kb NotI/PvuII and 360-bp XmnI/PstI fragments, and the 1.1-kb NotI/SacI and 360-bp XmnI/PstI fragments were ligated with the 9.6-kb NotI/PstI fragment from pSPB3LΔ2, pSPB3LΔ3, and pSPB3LΔ4, respectively. In the plasmid pSPB3LΔ3, the segment encoding amino acid residues 421–470 of the α1Δ subunit was deleted, and the cdon ATG for Met-471 was replaced with the cdon GTG for (Val). The resulting 5.6-kb SalI/SphI fragment was ligated with 3.6-kb HindIII (blunted)/SalI fragments and ligated with SacI, yielding plasmid pSPB3LΔ1 and pSPB3LΔ2, respectively. The 9.6-kb NotI/SacI fragment was digested with HindIII and SphI, ligated with T4 DNA polymerase, and digested with SalI. The resulting 5.6-kb SalI/SphI fragment was ligated with 3.6-kb HindIII (blunted)/SalI fragments and ligated with NotI, yielding plasmid pSPIC1 (originally pSPBC1A-1SH). The plasmid pSPIC1A, the segment encoding amino acid residues 1856–2725 of the α1Δ-1 (β1-α1) subunit was deleted, and the amino acid residues AFR-LRAAERG were attached to pSPB3LΔ1. The 170-bp BglII/NotI fragment was ligated with 5.8-kb XbaINheI, 2.6-kb NheIBglII, and 1.1-kb NotIXbaI fragment from pSPCB1-1 to yield pSPIC4. The plasmid carries cDNA encoding the α1Δ-1 α1α1Δ subunit with a deletion of the C-terminal amino acid residues 1925–2573.
The records were taken before (A mean and S.E. currents. The membrane was held at described previously (15). Also, the membrane potential recorded by the measured with the two-microelectrode voltage-clamp method as de-
scribed previously (15). Also, the membrane potential recorded by the measured with the two-microelectrode voltage-clamp method as de-

The oocytes were positioned in a recording chamber (1.0 ml in volume) and were perfused with a Ba2+ solution containing 40 mM Ba2+, 50 mM Na+, 2 mM K+, and 5 mM HEPES (pH 7.5 with methanesulfonic acid). Membrane currents through the expressed Ca2+ channels were measured with the two-microelectrode voltage-clamp method as described previously (15). Also, the membrane potential recorded by the potential electrode was monitored. The membrane was held at −80 or −100 mV, and step depolarizations were applied to activate the Ca2+ channels. Microelectrodes were filled with 3 M KCl, and those showing resistances of 0.5–1.5 mehmgs were used.

We noticed slow tail currents upon repolarization as shown in Fig. 1. In these cases, the time resolution of clamping was within 4 ms and the potential error was within 3% of the command pulse, indicating no serious space-clamping problems in characterizing Ca2+ channel currents.

Unless otherwise stated, statistical data were represented by the mean and S.E.

RESULTS

Functional Expression of the N-, P/Q-, and L-type Ca2+ Channels in Xenopus Oocytes—To establish a recombinant expression system, where current inhibition mediated by G-proteins can be reconstituted individually, HVA N-, P/Q-, and L-type Ca2+ channels were co-expressed in Xenopus oocytes by injection of cRNAs for three (α1, α2, and β1) Ca2+ channel subunits and the δ-opioid receptor (DOR). Their responses to the opioid peptide, Leu-enkephalin (Leu-EK), were examined by the two-microelectrode voltage-clamp technique.

Fig. 1 illustrates inward membrane currents recorded from Xenopus oocytes that were injected with the N-type α1B (Fig. 1, A and B), P/Q-type α1A (Fig. 1, C and D), and L-type α1C (Fig. 1, E and F) cRNA in combination with α2 and β1 subunits and DOR. As shown by the current-voltage (I-V) relationships in Fig. 1, step depolarizations from a holding potential of −80 mV produced long lasting inward currents at potentials more positive than −30 mV for oocytes injected with α1B (Fig. 1B) and α1A subunits (Fig. 1D) and at potentials positive to −50 mV with α1C (Fig. 1F).

As shown in Fig. 1A, inward currents recorded from oocytes implanted with α1B, α2, and DOR showed a time-dependent inactivation and a sensitivity to 0.1 μM ω-conotoxin GIVA (ω-Ctx), an N-type Ca2+ channel blocker. This current was not blocked by 0.3 μM ω-agatoxin IVA (ω-Aga), a P/Q-type Ca2+ channel blocker (n = 3), nor 10 μM nifedipine, a dihydropyridine (DHP)-derivative L-type Ca2+ channel blocker (n = 12). Application of Leu-EK (1 μM) to the bathing solution inhibited inward current from N-type channels within seconds (Fig. 1B).
The inhibited current displayed “kinetic slowing” of the current activation as well as an overall reduction in peak current (24, 25).

As shown in Fig 1, C and D, oocytes expressing α1A, α2, β1, and DOR exhibited inward currents which were blocked by 0.3 μM ω-Aga (Fig. 1C) but not by 0.3 μM ω-CTX (n = 3) nor 10 μM nifedipine (n = 9), consistent with previous findings. Application of Leu-EK to oocytes expressing α1A also displayed Ca2+ channel modulation, similar to that observed with α1B. Since DOR translates a signal to downstream effectors through activation of G-proteins (26), it is conceivable that the Leu-EK-induced inhibition of α1B and α1A currents is mediated by endogenous oocyte G-proteins.

Following the injection of L-type α1C cRNA in combination with α2, β1, and DOR cRNAs, inward currents were observed (Fig. 1, E and F), which were sensitive to 10 μM nifedipine (Fig. 1E) (18), but were not blocked by 0.3 μM ω-CTX (n = 3) nor 0.3 μM ω-Aga (n = 3). By contrast to the ω-CTX-sensitive N-type or ω-Aga-sensitive P/Q-type currents, these currents were not inhibited by Leu-EK (Fig. 1F).

Thus, based on the electrophysiological and pharmacological properties, α1B, α1A, and α1C channels functionally expressed in oocytes possessed the native characteristics of ω-CTX-sensitive N-type, ω-Aga-sensitive P/Q-type, and DHP-sensitive L-type Ca2+ channels, respectively. The Leu-EK-induced inhibition of inward Ba2+ currents was not appreciably observed when either DOR (n = 6) or the α1 subunit of Ca2+ channel (n = 6) cRNA was injected alone. In addition, ω-CTX- (n = 13), ω-Aga- (n = 3), and DHP (n = 15)-sensitive Ba2+ currents were not detectable without injection of α1B, α1A, and α1C subunit cRNAs.

Effects of Ga and Gβγ on the N- and P/Q-type Ca2+ Channels—To determine which arm of the G-protein complex contributes to regulation of N- and P/Q-type Ca2+ channels, respectively. Ga-protein-mediated inhibition of inward Ba2+ currents was not appreciably observed when either DOR (n = 6) or the α1 subunit of Ca2+ channel (n = 6) cRNA was injected alone. In addition, ω-CTX- (n = 13), ω-Aga- (n = 3), and DHP (n = 15)-sensitive Ba2+ currents were not detectable without injection of α1B, α1A, and α1C subunit cRNAs.

As detailed in the companion paper (16), agonist-induced inhibition of N-type α1B currents (Figs. 2A and 3A) and P/Q-type α1A currents (Fig. 2, A and C, B1) was further pronounced in oocytes injected with Ga or Gaβγ cRNA. In contrast, the inhibition of α1A and α1A channels was not potentiated in oocytes co-expressed with Gaβγ. However, Ba2+ currents recorded from oocytes injected with Ca2+ channel α1B, α2, and β1 subunits, DOR, and Gaβγ, were increased by a large conditioning depolarization to +80 mV without receptor stimulation (Fig. 2A, B3, open bar; also see Fig. 2 in the companion paper). This may indicate that the exogenous Gaβγ can inhibit the N-type Ca2+ channel by itself, therefore not requiring receptor-mediated activation of G-proteins. Prepulse facilitations were not prominent, but still significant, for the α1A channel when injected with Gaβγ (Fig. 4C, B1). Moreover, L-type α1C currents were never inhibited by the application of agonist nor facilitated by administration of a prepulse (Figs. 2A and 3A, CD).

Combination of α1B, α2, and β1 Subunits Is Required for the Inhibitory Regulations by Ga or Gaβγ—The α1B subunit of the Ca2+ channel forms the channel pore (4). As a result of this, N-type Ca2+ channel currents were not detectable without the injection of α1B subunit cRNA (n = 13). However, when the α1B subunit was expressed without the α2 and β1 subunits, Leu-EK still produced channel inhibition and slowing of the α1B currents via Gaα (n = 8). Moreover, the opioid-induced inhibition of α1B currents was larger in the absence of β1 subunit (n = 15) and did not change without the α2 subunit (n = 8) (27, 28). In addition, the prepulse facilitation of α1B currents mediated via Gaβγ (see Fig. 3) was also present without the α2 and β1 subunits (n = 5). These results suggest that both Ga and Gβγ can interact with the α1 subunit regardless of subunit composition and are able to produce channel modulation.
**FIG. 2.** Multiple structural domains in \(\alpha_{1B}\) channels for the inhibition mediated by \(G_{i3}\alpha\) and \(G_{b}\gamma\). A, left, schematic representation of mutant \(\alpha_{1B}\) and \(\alpha_{1C}\) channels and chimeric channels composed of \(\alpha_{1B}\) (hatched boxes) and \(\alpha_{1C}\) sequences (open boxes). Deletion or replacement of the C terminus and/or substitution of the intracellular loop between segment I and II (loop 1) were carried out in the two types of \(\alpha_i\) subunit of \(\text{Ca}^{2+}\) channels. Nomenclature is as follows: \(B3\), wild-type \(\alpha_{1B}\); \(CD\), wild-type \(\alpha_{1C}\); \(T\) or \(L\) or \(L1\), loop 1; \(\Delta\), deletion. Functional expression of \(\text{Ca}^{2+}\) channels is also indicated (+ and −). A, right, responsiveness of mutant and chimeric \(\alpha_i\) channels to 1 \(\mu\)M Leu-EK in oocytes implanted with \(DOR, \alpha_{1B}\), \(\alpha_{1C}\), \(b1a\) subunits in combination with \(G_{i3}\alpha\) (filled boxes), \(G_{b}\gamma\) (hatched boxes), or no exogenous G-protein (open boxes). Positive and negative responses represent inhibition and facilitation of channels, respectively. In oocytes from which endogenous \(\text{Ca}^{2+}\) currents were recorded, \(\alpha_{1B}\) subunit was not co-expressed (No exogenous \(\text{Ca}^{2+}\) channel). In other oocytes, wild-type, mutant, and chimeric \(\alpha_i\) subunits as indicated for each on the left side were co-expressed. The antisense oligonucleotide, AGO, was used. The responses to Leu-EK were measured (see Fig. 1 legend) and expressed as ratios of inhibition. The number of oocytes examined for each data are 4–68. B, representative current traces for the mutant \(\alpha_{1B}\) (B3T\(\Delta\)) and \(\alpha_{1C}\) (CDT\(\Delta\)) channels and the chimeric \(\alpha_{1B}/\alpha_{1C}\) (B3L\(\Delta\), B3LCD\(\Delta\), CDT\(\Delta\), CDT\(\Delta\), and CDB\(\Delta\)) channels in oocytes co-expressed with \(DOR, G_{i3}\alpha\), and \(\text{Ca}^{2+}\) channel \(\alpha_i\) and \(\beta_{1a}\) subunits. The pulse protocol was identical to that in Fig. 1 for \(\alpha_{1B}\) channels. Concentrations of Leu-EK, \(\omega\)-CTX, and nifedipine used were 1, 0.3, and 10 \(\mu\)M, respectively. The antisense oligonucleotide, AGO, was used.
the \( \alpha_{1c} \) chimera, CDLB3 (having a loop 1 region derived from \( \alpha_{1b} \)), restored the prepulse facilitation when \( \mathrm{G}_\beta_1\gamma_2 \) was co-expressed (Fig. 3A, CDLB3, open bar). Moreover, deletion of the C terminus of \( \alpha_{1b} \) enhanced the prepulse facilitation in oocytes co-expressed with \( \mathrm{G}_\beta_1\gamma_2 \) (Fig. 3A, B3T\( \Delta \)1, open bar).

When \( \mathrm{G}_\alpha \), instead of \( \mathrm{G}_\beta_1\gamma_2 \), was co-expressed, the agonist-induced inhibition of \( \mathrm{Ca}^{2+} \) currents was strengthened in wild-type \( \alpha_{1b} \) channels as compared with control oocytes, in which no exogenous G-proteins were co-expressed (Fig. 3A, B3, filled bar). This large inhibition was abolished by applying a large conditioning prepulse (filled circle). In chimeric \( \alpha_{1b} \) channels, B3LCD, such a potentiation of current inhibition by \( \mathrm{G}_\alpha \) was still detectable (Fig. 3A, B3LCD, filled bar) and almost entirely relieved by applying a prepulse (filled circle). Furthermore, deletion of the C terminus of B3LCD abolished the sensitivity to the agonist-induced inhibition with \( \mathrm{G}_\alpha \) (Fig. 3A, B3LCD\( \Delta \)1, filled bar). By contrast, the \( \alpha_{1c} \) chimera, CDTB3, having a C terminus derived from \( \alpha_{1b} \), acquired sensitivity to \( \mathrm{G}_\alpha \) and changes induced by prepulse without \( \mathrm{G}_\beta_1\gamma_2 \) in oocytes expressing \( \mathrm{G}_\beta_1\gamma_2 \) as shown in upper, are represented as \( \Delta \) Response.

To gain a clearer understanding of contributions of loop 1 in more detail, \( \mathrm{Ba}^{2+} \) currents through mutant \( \alpha_{1b} \) channels with four kinds of loop 1 deletions were studied (Fig. 3B). In the mutant channel, B3L\( \Delta \)2, with a deletion of amino acid residues 384–420 of \( \alpha_{1b} \) (Fig. 5A, \( \Delta \)2), the prepulse facilitation in oocytes co-expressed with \( \mathrm{G}_\beta_1\gamma_2 \) was diminished (Fig. 3B, B3L\( \Delta \)2, open bar). However, currents through the mutant channel, B3L\( \Delta \)3, with a deletion of amino acid residues 421–470 of \( \alpha_{1b} \) (Fig. 5A, \( \Delta \)3), were facilitated by a prepulse when \( \mathrm{G}_\beta_1\gamma_2 \) was co-expressed (Fig. 3B, B3L\( \Delta \)3, open bar). In both mutant channels, the potentiation by \( \mathrm{G}_\alpha \) of \( \mathrm{Leu} \)-EK-induced inhibition of currents was observed (filled bar). These characteristics of B3L\( \Delta \)2 indicate that deletion of loop 1, which nearly abolished interaction of the \( \alpha_{1b} \) subunit with \( \mathrm{G}_\beta_1\gamma_2 \), did not impair interactions with \( \mathrm{G}_\alpha \). On the other hand, we could not detect expression (\( n = 6 \)) of the mutants, B3L\( \Delta \)1 and B3L\( \Delta \)4, in which either a part of the loop 1 of \( \alpha_{1b} \) (amino acid residues 366–383, see Fig. 5A, \( \Delta \)1) or a part of the loop 1 of \( \alpha_{1b} \) that combines the regions covered by \( \Delta \)2 and \( \Delta \)3 (amino acid residues 384–470, see Fig. 5A) were deleted. Moreover, currents through the mutant channel, B3L\( \Delta \)2, were not detectable in the absence of \( \mathrm{Ca}^{2+} \) channel \( \beta \) subunit expression (31), this indicates that there may be another interaction site on the \( \alpha_{1b} \) channel for \( \beta \) subunits (32).

**Interaction Site on the P/Q-type \( \mathrm{Ca}^{2+} \) Channel for G-protein**—In order to determine the interaction site on the P/Q-type \( \mathrm{Ca}^{2+} \) channel for \( \mathrm{G}_\alpha \) and \( \mathrm{G}_\beta_1\gamma_2 \) procedures similar to those for \( \alpha_{1b} \) channels (Figs. 2 and 3) were applied to \( \alpha_{1a} \) channels (Fig. 4). In oocytes co-expressed with \( \mathrm{G}_\alpha \) or \( \mathrm{G}_\beta_1\gamma_2 \) together with...
DOR and Ca\(^{2+}\) channel (\(\alpha_1, \alpha_2\) and \(\beta_{1a}\) subunits), deletion of the C terminus of \(\alpha_{1A}\) (amino acid residues 2015–2273) reduced the sensitivity to Leu-EK (Fig. 4A, B1T2) as compared with the wild-type \(\alpha_{1A}\) (B1). This stands clearly in contrast to the mutant \(\alpha_{1B}\) channel (B3T2), in which deletion of the C terminus alone did not influence the sensitivity to Leu-EK (Fig. 2A).

In addition, the chimeric \(\alpha_{1C}\) channel (CDTB1), in which the C terminus of \(\alpha_{1C}\) (amino acid residues 1524–2127) was replaced by that of \(\alpha_{1A}\) (amino acid residues 1838–2424), acquired sensitivity to Leu-EK (Fig. 4A, CDTB1), whereas the wild-type \(\alpha_{1C}\) channel was not affected by Leu-EK (Figs. 2A and 3A). Another chimeric \(\alpha_{1C}/\alpha_{1A}\) channel (CDB1), in which the N terminus of \(\alpha_{1C}\) (amino acid residues 1–777) substituted for that of \(\alpha_{1A}\) (amino acid residues 1–707), still exerted sensitivities to Leu-EK (Fig. 4A, CDB1), as shown in Fig. 4B, currents through the B1T2 and CDTB1 channels were comparable to those through the wild-type \(\alpha_{1A}\) and \(\alpha_{1C}\) channels (Fig. 1), and the CDTB1 currents were blocked by nifedipine.

Next, the mutant (B1T2) and chimeric (CDTB1) \(\alpha_{1A}\) channels were further characterized using a double-pulse protocol. Fig. 4C illustrates responses to application of a prepulse and Leu-EK by these channels and also demonstrates changes induced by prepulse without Leu-EK in oocytes expressing \(\alpha_{1A}\) as well as differences in the response to Leu-EK between oocytes with and without expression of \(\alpha_{1C}\). The potentiation by \(G_{3\alpha}\) of Leu-EK-induced inhibition observed in the wild-type

![Diagram](Fig. 4. Regulation of P/Q-type (\(\alpha_{1A}\)) channels by Go and G\(\beta\gamma\)). A, left, schematic representation of mutant \(\alpha_{1A}\) channels and chimeric channels composed of \(\alpha_{1A}\) (filled boxes) and \(\alpha_{1C}\) sequences (open boxes). Nomenclature is given in the legend of Fig. 2A, except that B1 denotes wild-type \(\alpha_{1A}\). Functional expression of Ca\(^{2+}\) channels is also indicated (+ and –). A, right, responsiveness of mutant \(\alpha_{1A}\) and chimeric \(\alpha_{1A}/\alpha_{1C}\) channels to 1 \(\mu\)M Leu-EK in oocytes implanted with DOR, \(\alpha_1, \alpha_2\), and \(\beta_{1a}\), in combination with \(G_{3\alpha}\) (filled boxes), \(G_{\beta_{1a}}\) (hatched boxes), or no exogenous G-protein (open boxes). In oocytes from which endogenous Ca\(^{2+}\) currents were recorded, \(\alpha_1\) subunit was not co-expressed (No exogenous Ca\(^{2+}\) channel). In other oocytes, wild-type, mutant, and chimeric \(\alpha_1\) subunits as indicated for each on the left side were co-expressed. The antisense oligonucleotide, AGO, was used. The responses to Leu-EK were measured (see Fig. 2A) and expressed as ratios. The number of oocytes examined for each data are 4–18. B, representative current traces for the mutant (B1T2) and chimeric (CDTB1) \(\alpha_{1A}\) channels in oocytes implanted with DOR, \(G_{3\alpha}, \alpha_2, \alpha_2\), and \(\beta_{1a}\), in combination with each mutant or chimeric \(\alpha_1\). The pulse protocol was identical to that as in Fig. 1 for the \(\alpha_{1A}\) channel. Concentrations of Leu-EK and nifedipine used were 1 and 10 \(\mu\)M, respectively. The antisense oligonucleotide, AGO, was used. C, comparisons of wild-type, mutant, and chimeric \(\alpha_{1A}\) channels with respect to the Leu-EK-induced inhibition via \(G_{3\alpha}\) and to the prepulse facilitation via \(G_{\beta_{1a}}\). The responses of three different channel types, as indicated, to 1 \(\mu\)M Leu-EK (horizontal bars), prepulse (open circles), or both (filled circles) were measured (see Fig. 3A) and expressed as ratios. The antisense oligonucleotide, AGO, was used. The number of oocytes examined for each data are 4–18.

Interaction of Ca\(^{2+}\) Channel with Go and G\(\beta\gamma\)


**INTERACTION OF Ca2⁺ CHANNEL WITH Ga AND GBγ**

![Diagram](image)

**FIG. 5.** Schematic representation of the sites on α1b subunit for making the deletion mutants. A, positions of the deletion (LΔ1, LΔ2, LΔ3, and TΔ1), the loop 1, and the C terminus are indicated by the number of the amino acid residues for α1b subunit (33) and α1α (Bl-1 α1) subunit (20) in parentheses. The deletion sites are indicated by the crossing bars, and the cytoplasmic side below the horizontal lines. The asterisk denotes the phosphorylation sites for protein kinase C (10). B, mutations introduced into the α1b and α1α subunits. The numbers of amino acid residues deleted are indicated.

α1α (Fig. 4C, B1, filled bar) almost disappeared in the mutant α1αΔ1B, B1Δ2, having the deletion of C terminus (Fig. 4C, B1Δ2Δ, filled bar). By contrast, the α1α/α1α chimera, CDTB1 (having the C terminus derived from α1αΔ1), conferred Leu-EK sensitivity via Gbg3 (Fig. 4C, CDTB1, filled bar). In both the wild-type α1α and the chimera CDTB1, the prepulse did not abolish the potentiation of Leu-EK-induced inhibition via Gbg3 (filled circles). In addition, when Gbg3 was co-expressed instead of Gbg3, a small facilitation by prepulse was observed in the wild-type α1α (Fig. 4C). In the mutant (B1Δ2) and chimeric (CDTB1) channels, prepulse facilitation was not detected.

**DISCUSSION**

In the present study, the ω-CTX-sensitive N-type (α1β) and ω-Aga-sensitive P/Q-type (α1Α) Ca2⁺ channels were functionally expressed in Xenopus oocytes, an *in vivo* expression system. As described, we found that Gbg3 co-expressed in oocytes mediated receptor agonist-induced inhibition of N-type α1b and P/Q-type α1α channels. On the other hand, a depolarizing prepulse relieved current inhibition caused by the Gbg3 complex, and the facilitatory effects were more pronounced in α1b than in α1α. Because responsiveness of the α1b and α1α channels to the inhibition mediated by Gbg3 and Gbg3Δ was maintained even in the absence of the Ca2⁺ channel auxiliary subunits α2 and β1, the α1 subunit should bear the interaction sites for both the Gα subunit and the Gβγ dimer. Finally, we defined loop 1 as an interaction site for Gβγ and the C terminus of α1b and α1α for Gα, based on the responses of mutant and chimeric channels to Gα and Gβγ.

**The Native Type α1p, α1α, and α1c Channels Expressed in Xenopus Oocytes** —The electrophysiological and pharmacological properties of the α1b, α1α, and α1c channels determined were identical to those of the N, P/Q-, and L-type Ca2⁺ channels described previously (18, 20, 33). This indicates that α1b (N-type), α1α (P/Q-type), and α1c (L-type) Ca2⁺ channels were functionally expressed with the Ca2⁺ channel α2 and β subunits in Xenopus oocytes. When DOR was further co-expressed, α1b and α1α channel currents, but not α1c channel currents, were inhibited within seconds when stimulated by Leu-EK. It is likely that agonist-induced excitations of α1b and α1α channels are mediated by endogenous oocyte G-proteins that are coupled to the receptor, because the inhibitions of α1b and α1α channels were reduced when the antisense oligonucleotide AGO against *Xenopus* Gbg3 was injected (16).

The Loop 1 of α1b Channel as an Interaction Site for Gβγ—When Gbg3 was co-expressed, the Leu-EK-induced inhibition was not potentiated in either α1b or α1α channels. In the case of N-type α1b, however, a depolarizing prepulse to +80 mV facilitated the currents in the absence of the receptor agonist, suggesting that the exogenous Gbg3 inhibits the α1b channel by itself (8, 9). Thus, the difference between the current traces before and after the prepulse should correspond to an α1b current component that is mainly inhibited by exogenous Gbg3.

A diminished response to prepulse application was found for an α1b channel (B3LCD) chimerized with the α1c loop 1 and a mutant α1b (B3LΔ2) with a partial deletion of the loop 1 (including the binding site for Ca2⁺ channel β subunit) (see Fig. 5A), when Gbg3 was co-expressed. On the other hand, an α1c channel (CDLB3) chimerized with the α1b loop 1 conferred properties of facilitation by prepulse on this channel when expressed with Gbg3. Taken together, these findings indicate that the loop 1 plays an essential role for the interaction of the α1b channel with Gbg3. This is consistent with recent evidence that has shown the direct binding of Gbg3 to the loop 1 of α1b (10, 11, 16). The prepulse facilitation could not be abolished completely by deleting the loop 1 of α1b (B3LΔ2), whereas it...
could be totally abolished by replacing the whole loop 1 of α1B with that of α1B (B3LCD). As described in the companion paper (16), a loop 1 peptide (PL1) corresponding to amino acid residues 366–384 of α1B almost abolished the prepulse facilitation. This means that the interaction site on the α1B loop 1 for Gβγ might cover both the deletion site in B3LΔ2 and the region determining PL1 (10, 11). The flanking region of the α1B loop 1 (amino acid residues 421–470) including the phosphorylation sites for protein kinase C (10) was not critical for the interaction between the channel and Gβγ as examined by B3LΔ3, a mutant with a partial deletion of the α1B loop 1 (see Fig. 5A).

On the other hand, a mutant α1B channel devoid of the normal C terminus (B3TΔ1) never failed to induce prepulse facilitation via Gβγγ. Inversely, the α1C channel chimerized with the α1B C terminus (CDTB3) did not facilitate the current by prepulse in the presence of Gβγγ. These findings indicate that the C terminus of α1B channel is not involved in the prepulse facilitation via Gβγγ.

In the case of the P/Q-type α1A channel, prepulse facilitation was not as prominent as observed in the α1B channel when Gβγγ was co-expressed (16). Nonetheless, the prepulse facilitation via Gβγγ appeared to be abolished in a mutant α1A channel (B1TΔ2) that contained a deletion of the C terminus. This is in contrast to a similar α1B mutant (B3TΔ1), in which the prepulse facilitation via Gβγγ was rather enhanced. However, an α1C channel (CDTB1) chimerized with the α1A C terminus did not confer facilitation by prepulse. These results suggest that the contribution of the C terminus to channel inhibition via Gβγγ is small, although the possibility of interaction of the α1A C terminus with Gβγγ (34) has not been excluded.

The C Termi of α1B and α1A Channels as an Interaction Site for Gα—Receptor stimulation by agonist is known to catalyze activation of Go and lead to dissociation of the Goβγ heterotrimer (1). In fact, application of a prepulse did not facilitate N-type α1B channels when co-expressed with Gα10 and DOR, whereas DOR was stimulated by Leu-EK. The potentiating action of Gα10 on the agonist-induced inhibition of α1B channels was abolished by application of a large conditioning prepulse. This suggests that exogenous Gα, unlike Gβγγ, does not influence the α1A channel by itself and stays in its inactive form. It appears, therefore, that potentiation of agonist-induced inhibition via exogenous Gα results from the interaction of the channel with activated exogenous Gα and, probably, with an endogenous Gβγγ dissociated from the Gα. This idea is further evidenced by the observation for mutant (B3TΔ1) and chimera (B3TCD) α1B channels, in which loss of the C terminus (a possible interaction site for Gα10, see below) did not affect the potentiation of inhibition via Gα10. However, a further loss of the loop 1, an interaction site for Gβγγ of the mutant B3TΔ1 α1B channel (B3LΔCD1) eliminated the potentiation of inhibition via Gα10 as well as the prepulse facilitation via Gβγγ. Moreover, an α1B channel chimerized with the α1C loop 1 (B3LCD), in which the interaction site exclusively for Gβγγ was lost, retained the potentiation of inhibition via Gα10 but no longer the prepulse facilitation via Gβγγ. These results indicate that there are two distinct interaction sites, namely loop 1 and the C terminus, for Gα and Gβγγ on N-type Ca2+ channels and that the two sites regulate the channel activity independently when they receive inhibitory signals from Gα and Gβγγ. This independence of loop 1 and the C terminus in the α1B channel modulation is supported by the observation that the Gα10-dependent potentiation of inhibition was not affected by single application of the loop 1 peptide (PL1) or a C-terminal peptide (PB3T4) inside the oocyte but abolished by simultaneous application of both of them (16).

As in the case of B3LCD described above, mutant α1B channels devoid of the normal loop 1 (B3LΔ2 and B3LΔ3) never failed to potentiate the current inhibition in response to Leu-EK via Gα10. Inversely, the α1C channel chimerized with the α1B loop 1 (CDLB3) did not potentiate the inhibition via Gα10. These findings indicate that there is an interaction site for Gα outside the loop 1 of α1B channel (14).

The α1B channel chimerized with the α1C loop 1 (B3LCD), which was devoid of the interaction site for Gβγ, did not impair Gα10-dependent potentiation in the inhibitory response to Leu-EK unless its C terminus was deleted (B3LCDTΔ1). Contrary, an α1C channel chimerized with the α1B C terminus (CDTB3) potentiated the inhibition via Gα10. Together, the results indicate that the C-terminal segment of α1B is essential for the interaction with Gα.

In the case of the P/Q-type α1A channel, Leu-EK-induced inhibition was markedly potentiated when co-expressed with Gα10, similar to the α1B channel. In contrast to the α1B channel, prepulse failed to abolish potentiation of inhibition via Gα10. Furthermore, Gα10-dependent potentiation of inhibition of the α1A channel was impaired only when the C terminus was deleted (B1TΔ2). This is probably due to a minor contribution of Gβγ to the potentiation of inhibition by agonist, despite the direct binding of Gβγ to the loop 1 of α1A (11, 16). In fact, the potentiation of inhibition via Gα was abolished by intracellular application of a C-terminal peptide (PPQT1) alone (16). Moreover, an α1C channel chimerized with the α1A C terminus (CDTB1) potentiated the Leu-EK-induced current inhibition via Gα10. These findings indicate that, as in the case of α1B, the C terminus of α1A is also essential for the interaction with Gα and that, in contrast to α1B, the loop 1 of α1A appears not to be essential in the channel regulation by G-proteins.

In conclusion, N- and P/Q-type Ca2+ channels are differentially regulated by Gα and Gβγγ in a way that prepulse preferentially facilitates N-type and that the C terminus and the loop 1 of N-type are equally involved in agonist-induced inhibition, whereas the C terminus of P/Q-type is mainly involved. Further studies using an in vitro binding assay will be necessary to determine the direct interaction of Gα and Gβγγ with N-type α1B and P/Q-type α1A Ca2+ channels.

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