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

II. EVIDENCE FOR DIRECT BINDING*

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The present study was designed to obtain evidence for direct interactions of G-protein \(\alpha\) (Go) and \(\beta\gamma\) subunits (G\(\beta\gamma\)) with N- (\(\alpha_{1B}\)) and P/Q-type (\(\alpha_{1A}\)) Ca\(^{2+}\) channels, using synthetic peptides and fusion proteins derived from loop 1 (cytoplasmic loop between repeat I and II) and the C terminus of these channels. For N-type, prepulse facilitation as mediated by G\(\beta\gamma\) was impaired when a synthetic loop 1 peptide was applied intracellularly. Receptor agonist-induced inhibition of N-type as mediated by Go was also impaired by the loop 1 peptide but only when applied in combination with a C-terminal peptide. For P/Q-type channels, by contrast, the Go-mediated inhibition was diminished by application of a C-terminal peptide alone. Moreover, in vitro binding analysis for N- and P/Q-type channels revealed direct interaction of Go with C-terminal fusion proteins as well as direct interaction of G\(\beta\gamma\) with loop 1 fusion proteins. These findings define loop 1 of N- and P/Q-type Ca\(^{2+}\) channels as an interaction site for G\(\beta\gamma\) and the C terminus for Go.

High voltage-activated (HVA) Ca\(^{2+}\) channels are negatively regulated by guanine nucleotide-binding regulatory proteins (G-proteins) in various neuronal preparations, including neoroblastoma \(\times\) glioma hybrid NG108-15 cells (1, 2), dorsal root ganglion neurons (3, 4), sympathetic neurons (5), and rat pituitary GH\(_2\) cells (6). This response appears to be controlled by a membrane-delimited mechanism via pertussis toxin (PTX)-sensitive G-proteins, in which the Go subunit has been shown to mediate an inhibitory signal to HVA Ca\(^{2+}\) channels. The primary structures of multiple subtypes of G-protein \(\alpha\) subunits (Go) including Go\(_{1A}\) have been deduced by molecular cloning and sequencing of their cDNAs. These studies revealed very similar but distinct amino acid sequences for each subtype cloned (7). It remains to be seen, however, which subtypes of Go preferentially interact with HVA Ca\(^{2+}\) channels such as N- and P/Q-types. In the previous study using mutant and chimeric channels expressed in Xenopus oocytes (8), our results provided evidence that the cytoplasmic I-II loop (referred to as “loop 1” in the present study) of N-type (\(\alpha_{1B}\)) Ca\(^{2+}\) channels is a regulatory site for the G-protein \(\beta\gamma\) dimer (G\(\beta\gamma\)) and the C termini of P/Q- (\(\alpha_{1A}\)) and N-type Ca\(^{2+}\) channels for Go. However, this does not answer the question as to whether Go, as well as G\(\beta\gamma\) (9, 10), directly interact with these Ca\(^{2+}\) channels.

To address these issues, we have expressed \(\alpha_{1B}\) and \(\alpha_{1A}\) HVA Ca\(^{2+}\) channels in Xenopus oocytes, in which the effects of intracellularly applied loop 1 and C-terminal peptides derived from \(\alpha_{1B}\) and \(\alpha_{1A}\) were investigated. Furthermore, a direct association of Go and G\(\beta\gamma\) with these Ca\(^{2+}\) channels was determined by in vitro binding using glutathione S-transferase (GST) proteins fused with the loop 1 and the C-terminal segments of \(\alpha_{1B}\) or \(\alpha_{1A}\). These results, taken together with the findings of the previous study (8), define the interaction sites of Go and G\(\beta\gamma\) within Ca\(^{2+}\) channels.

**EXPERIMENTAL PROCEDURES**

In Vitro Transcription—The 1.8-kilobase pair NcoI/SalI fragment containing the entire coding region of the \(\beta_2\)-adrenergic receptor (\(\beta_2\AR\)) (11) was inserted into the HindIII site of the pSP2AR vector (12), to yield pSP12AR. The \(\beta_2\AR\) cDNA was kindly provided by Dr. Robert J. Lefkowitz. The pSP1A, pSP2A, pSP72, pSP65, and pSP64 recombinant plasmids carrying the entire protein-coding sequences of Go (Go\(_{1A}\), Go\(_{1B}\), Go\(_{1C}\), Go\(_{1D}\), and Go\(_{1E}\)), G\(\beta_1\), G\(\beta_2\), G\(\beta_3\), \(\delta\)-opioid receptor (DOR), and the Ca\(^{2+}\) channel \(\alpha_{1B}\), \(\alpha_{1A}\), \(\alpha_{1C}\), \(\alpha_{1D}\), and \(\beta_{1}\) subunits were described previously (8, 12–16). Nucleotide sequence analyses revealed that the deduced amino acid sequence of Go\(_{1D}\) was similar to that reported (17) except that Glu-99, Ala-113, Met-119, Thr-280, and Glu-281 were determined as Ser (TCC), Thr (ACG), Val (GTG), Ile (ATG), and His (CAC), respectively, in our clone, pG2ox (15).

cDNAs specific for \(\alpha_{1A}\), \(\alpha_{1A}\), \(\alpha_{1C}\), \(\alpha_{1D}\), and \(\beta_{1}\) subunits of the Ca\(^{2+}\) channel, DOR, \(\beta_2\AR\), and six isoforms of Go, G\(\beta_1\), or G\(\gamma_2\) were synthesized in vitro using the MEGAscript SP6 kit (Ambion).
Functional Expression of Ca\textsuperscript{2+} Channels in Xenopus Oocytes—According to the methods described in the companion paper (8), Xenopus oocytes were injected with cRNAs and subjected to electrophysiological measurements. cRNAs were used either with 0.3 \mu{g}/\mu{l} \alpha_1, \alpha_\text{AR}, \text{or} \alpha_\text{IR} \text{cRNA in combination with 0.2 \mu{g}/\mu{l} \alpha_2 \text{cRNA and 0.1 \mu{g}/\mu{l} \beta_3 \text{cRNA}}. The pipette solution contained 110 m\text{M} (NG\text{I3-6} \text{and} NG\text{I3-13}) \text{and} 90 \text{mM} K\text{injected with the antisense AGO, were bathed in a depolarizing solution consisting of 90 mM K}^+ \text{injected with the antisense AGO, were bathed in a depolarizing solution containing 50 mM BaCl}_2, \text{3 mM NaCl, 1 mM MgCl}_2, \text{5 mM CaCl}_2, \text{20 mM glucose, 2 mM tetrodotoxin, and 10 mM HEPES (pH 7.24). The patch electrodes contained solutions of the following composition: 150 mM CsCl, 1 mM MgCl}_2, 10 mM EGTA, 1 mM ATP, 10 mM HEPES (pH 7.3). Muscarinic acetylcholine (ACH) receptors were stimulated by a focal application of 1 mM Ach (3 \mu{l}). In experiments in which PTX was used, the cells were preincubated with the toxin (500 ng/ml) for 12–14 h prior to the measurements. The electrophysiological experiments were carried out at approximately 30 °C. Statistical data were represented by the mean and S.E.

G\text{a} \text{and} G\text{bg} \text{Signings}—The 1.6-kilobase pair Sr/UsaI fragment (encoding amino acid residues 1912–2339 of the \alpha_\text{b} \text{subunit}) and the 1.4-kilobase pair Sca/UbaI fragment (encoding amino acid residues 1975–2424 of the \alpha_\text{b} \text{subunit}) were excised from the plasmids pSPB3 (16) and pSPCBI-2 (14) and fused in-frame to the GST coding sequence in pGEX-2T (Amersham Pharmacia Biotech) to produce GST-fusion proteins of C terminus (GST-B3T and GST-B1T), respectively. Similarly, to produce GST fusion proteins of loop 1 (GST-B3L1 or GST-B1L1), the 380-base pair fragment amplified by polymerase chain reaction (PCR), encoding either amino acid residues 361–483 of the \alpha_\text{b} \text{subunit} or 385–489 of the \alpha_\text{b} \text{subunit}, was fused in-frame. The sets of primers for PCR were GGGAAATTCGTAGGCGCGGAGA and TCTTGCCTCTCACCAGGGCC for \alpha_\text{a} \text{GST fusion proteins and glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) at 4 °C for 5 h and washed five times with 40 volumes of phosphate-buffered saline containing 1% Triton X-100. Approximately 10 \mu{g} of GST, or GST-fusion proteins, bound to beads were used in each binding assay.

Ten micrograms of purified bovine brain G\text{a} (26) and G\text{b} complex (27) were incubated at 4 °C for 12 h with the beads that had been washed four times with 20 mM Tris-Cl (pH 7.5) supplemented with 0.2% sodium deoxycholate and 0.6% sodium cholate, respectively. These buffers contained 20 mM Tris-HCl (pH 8), 0.1 mM EDTA, 0.5 mM dithiothreitol, 20 mM NaPO\text{4}, and a mixture of protease inhibitors (2.5 \mu{g}/ml pepstatin, 2 \mu{g}/ml phenylmethylsulfonfluoride, 0.02 mg/ml leupeptin, and 0.5 mM benzamidine). Then, the beads were washed with 40 volumes of the same buffer solutions and denatured by heating to 90 °C for 3 min in 200 \mu{l} of a solution of 50% acetic acid and 2% sodium dodeyl sulfoxide. The beads were then solubilized in SDS-PAGE loading buffer. Proteins (70 \mu{l}) were separated by SDS-PAGE and transferred to Immobilon membrane (Millipore) for Western blotting and detection by the ECL system (Amersham Pharmacia Biotech) as described previously (28). The antibody, G\text{AB}, raised against the C-terminal decapeptide of G\text{a} (FG\text{B}) (29) was kindly provided by Dr. Tatsuya Haro, and the antibodies, K-20 and M-14, against G\text{a} and G\text{bg}, respectively, were purchased from Santa Cruz. The first antibody when used was diluted appropriately to reduce nonspecific reactivity, and the second antibody (bionitlated anti-rabbit Ig, Amersham Pharmac Biotech) and the peroxidase-streptavidin (Vector) were both diluted at 1:2000. Each incubation was for 1 h at room temperature. The decapeptide FG\text{B} (Sawady, Japan) was synthesized based upon the amino acid sequence corresponding to the amino acid residues 345–354 of G\text{a} (29). The peptide antigens sc-387P (for K-20) and sc-261P (for M-14) were also purchased from Santa Cruz. The GST fusion proteins (GST-B3T, GST-B1T, GST-B3L1, and GST-B1L1) and GST proteins were tested more than three times for their ability to bind G\text{a} and G\text{bg}. There was no essential difference between G\text{a} and G\text{bg} in detecting specific bindings of G\text{a} to the fusion proteins.

RESULTS

Effects of Ga on the N- and P/Q-type Ca\textsuperscript{2+} Channels—In the previous study (8), N-type (\alpha_\text{N}), P/Q-type (\alpha_\text{NPQ}), and L-type (\alpha_\text{CL}) \text{Ca}^\text{2+} \text{channels were shown to be functionally expressed in Xenopus oocytes, and \alpha_\text{N}, \alpha_\text{NPQ}, and \alpha_\text{CL} \text{channels were negatively}
regulated by $G_{a\alpha}$ and $G_{b\beta,\gamma}$. To determine further which $G_{a}$ isoforms regulate HVA Ca$^{2+}$-channels, either Ga cRNA (for six different isoforms: $G_{i1}, G_{i2}, G_{i3}, G_{o1}, G_{o2}$, and $G_{o3}$) or $G_{i1}$ plus $G_{i2}$ cRNAs were injected into oocytes in combination with the Ca$^{2+}$ channel $a_1$ ($a_{1B}$ or $a_{1A}$), $a_2$, and $\beta_1$ subunits cRNAs and receptor (DOR or $d_2A$) cRNA. When $G_{a}$ was expressed, $\beta_2$AR was co-expressed, and the receptor was stimulated by 1 $\mu$m isoproterenol, an agonist of the $\beta$-adrenergic receptor.

Agonist-induced inhibitions of $\alpha_{1B}$ channels were less pronounced in oocytes co-injected with $G_{i1}, G_{i2}, G_{o1},$ or $G_{b\beta,\gamma}$ cRNA, as compared with control oocytes injected with Ca$^{2+}$ channel subunits ($a_{1B}, a_2$, and $\beta_1$) and DOR (Fig. 1A, upper). Also, Leu-EK-induced inhibition was diminished in oocytes injected with $G_{a}$ but not with $G_{a}$ nor $G_{o}$. These observations are consistent with the fact that $G_{a}$ is PTX-sensitive, and $G_{o}$ and $G_{a}$ are PTX-insensitive G-proteins (30). Because a PTX-insensitive component of the response was increased in oocytes co-expressed with the PTX-sensitive $G_{a}$ or $G_{a}$, it was presumed that a maximal inhibition of the $\alpha_{1B}$ channel was already attained by endogenous oocyte G-proteins and that introduced $G_{a}$ or $G_{a}$ was capable of replacing endogenous G-proteins when exerting current inhibition. Therefore, in order to unmask the effects of exogenous Ga, the antisense oligonucleotide, AGO, directed against mRNAs encoding Xenopus $G_{a}$ (8), was injected prior to the electrophysiological studies. As expected, Leu-EK-induced inhibition of $\alpha_{1B}$ channels in control oocytes (without exogenous Ga subtypes) was reduced by 42.3 $\pm$ 7.4% ($n = 25$) in the presence of antisense oligonucleotide AGO. As a result, the hierarchy of exogenous Ga subtypes in inhibiting $\alpha_{1B}$ channels could be more clearly recognized (Fig. 1A, lower) when compared with antisense-free control experiments (Fig. 1A, upper). Sense oligonucleotide, SGO, to Xenopus $G_{a}$ had no effects on the inhibition of $\alpha_{1B}$ channels ($n = 6$).

After antisense treatment, the agonist-induced inhibition of N-type $a_{1B}$ currents was further pronounced in oocytes injected with $G_{a}$, $G_{i1}, G_{o1},$ or $G_{o}$ cRNA (Fig. 1A, lower). Here, the action of $G_{a}$ would not be associated with adenylate cyclase, since the injection of 50 nl of 10 $\mu$m cyclic AMP ($n = 3$) or 10 units/ul catalytic subunit of PKA ($n = 3$) failed to influence $\alpha_{1B}$ currents. Moreover, the pretreatment with 100 $\mu$m H7, a inhibitor of cyclic nucleotide-dependent protein kinase and protein kinase C, did not affect the agonist-induced inhibition of $\alpha_{1B}$ currents ($n = 6$). However, in the case of L-type $a_{1C}$ channels, the catalytic subunit of PKA increased their current amplitude by 82.8 $\pm$ 10.9% ($n = 6$). In oocytes injected with cRNAs for Ca$^{2+}$ channel subunits ($a_{1B}, a_2,$ and $\beta_1$) and $\beta_2$AR, isoproterenol-induced inhibition of $\alpha_{1B}$ channels was 17.5 $\pm$ 1.2% ($n = 45$).

To exclude further the possibility that such diffusible second messengers might be involved in the effects of G-proteins in potentiating the agonist-induced inhibition of N-type $\alpha_{1B}$ channels, cell-attached patch recordings were performed (see “Experimental Procedures”). In oocytes implanted with N-type Ca$^{2+}$ channel subunits, DOR and $G_{a}$, multiple single channel currents through a membrane patch were suppressed by Leu-EK applied to the patch, but the application to the rest of the cell membrane was ineffective ($n = 4$). The extent of inhibition was 48.0 $\pm$ 8.0% ($n = 4$), which was comparable to that observed for the whole cell currents of oocytes (Fig. 1A, lower). Thus, Leu-EK has to be applied directly to the recording membrane patch to induce current inhibition, indicating that the Leu-EK-induced inhibition of $\alpha_{1B}$ channels associated with $G_{a}$ employs a membrane-delimited pathway as predicted in native neurons.

As shown in Fig. 1C, the extent to which Leu-EK inhibited $\alpha_{1B}$ channels was dependent on the amount of Ga cRNA injected. The rank order of efficiency among Ga subtypes examined was $G_{a} > G_{a} > G_{o}$ ($n = 4$). The agonist-induced current inhibition was considerably reduced when a low concentration (15 ng/ml) of $G_{a}$ cRNA was applied. By contrast, oocytes co-injected with $G_{i1}$ or $G_{i2}$ cRNA showed no effect on the Leu-EK-induced current inhibition, whereas oocytes injected with $G_{o}$ showed an attenuating effect (Fig. 1A, lower).

Unlike N-type $\alpha_{1B}$ channels (Fig. 1A, upper), P/Q-type $\alpha_{1A}$ channels revealed more conspicuous intensifications of the agonist-induced inhibition by exogenous Ga, probably a result of weak masking effects of endogenous G-proteins (Fig. 1B, upper). The agonist-induced inhibition of currents was potentiated in those oocytes co-expressed with $G_{i1}, G_{i2}, G_{o1}, G_{o2}$, or $G_{o3}$ but not with $G_{a}$ or $G_{o}$, regardless of antisense oligonucleotide (AGO) injection (Fig. 1B), although AGO did attenuate the Leu-EK-induced inhibition of $\alpha_{1B}$ channels by 23.2 $\pm$ 8.6% ($n = 6$), as compared with its effect on $\alpha_{1B}$ channels (42.3 $\pm$ 7.4%, $n = 25$). In addition, the agonist-induced inhibition of $\alpha_{1A}$ channels was diminished in PTX-treated oocytes co-expressed with $G_{i1}$ (Fig. 1B, upper). In oocytes co-expressed with the PTX-insensitive $G_{a}$, the blockade of agonist-induced inhibition of $\alpha_{1B}$ channels by PTX was at the same level as in control oocytes absent of exogenous Ga. This finding is consistent with the observation that the agonist-induced inhibition of $\alpha_{1A}$ currents was not potentiated in oocytes co-expressed with $G_{a}$. The inhibition of $\alpha_{1A}$ channels was not potentiated in oocytes co-expressed with $G_{i1}$ similar to $\alpha_{1B}$ channels.

By contrast, the agonists never evoked an inhibition of L-type $\alpha_{1C}$ currents in oocytes expressed with the Ca$^{2+}$ channel $\alpha_{1C}, a_2,$ and $\beta_1$ subunits and the receptor (DOR or $d_2A$) in combination with either of the six different isoforms of Ga ($n = 6$) or $G_{i2}$ ($n = 6$), even if the concentration of Ga-cRNA injected was increased to 150 ng/ml ($n = 4$).

To examine whether specifications of Ga-mediated inhibition of HVA Ca$^{2+}$-channels are reproducible in neuronal cells, we investigated the mechanism by which native HVA Ca$^{2+}$-channels are regulated by Ga in NG108-15 neuroblastoma-glioma hybrid cells. NG108-15 cells express N- and L-type Ca$^{2+}$-channels (31, 32) and DOR (24) and muscarinic ACh receptors (21) which inhibit the Ca$^{2+}$ channel activity. These native HVA Ca$^{2+}$-channels were sensitive to 10 $\mu$m nifedipine ($n = 10$) or 0.3 $\mu$m $\omega$-conotoxin GVIA ($\omega$-CTX) ($n = 6$), but not to 0.3 $\mu$m $\omega$-agatoxin IVA ($n = 3$). Puff application of 1 mM ACh inhibited HVA Ca$^{2+}$ currents in the presence of nifedipine ($n = 5$). On the other hand, ACh did not inhibit HVA Ca$^{2+}$ currents in the presence of both nifedipine and $\omega$-CTX ($n = 4$). These results indicate that ACh inhibits N-type Ca$^{2+}$-channel currents in NG108-15 cells. When exogenous Ga isoforms were stably expressed in NG108-15 cells according to the procedures described previously (20), the ACh-induced inhibition of HVA Ca$^{2+}$-currents was potentiated in the clones transfected by the exogenous $G_{i1}$ and $G_{a}$, but not by $G_{i2}$ or $G_{o2}$ (Fig. 1D). Thus, the specifities of Ga in inhibiting native N-type Ca$^{2+}$-channels were similar to those observed in Xenopus oocytes. PTX (500 ng/ml, a supramaximal dose) impaired the potentiation by $G_{i1}$ and $G_{a}$ of the ACh-induced inhibition of Ca$^{2+}$-channels (Fig. 1D).

These results indicate that the N-type $\alpha_{1B}$ channel is negatively regulated by $G_{i1}, G_{i2}, G_{o1}, G_{o2}$, and $G_{a}$ and that the P/Q-type $\alpha_{1A}$ channel is also negatively regulated by $G_{i1}, G_{i2}, G_{o1}, G_{o2}$, and $G_{a}$. It is further suggested that the N- and P/Q-type
Ca\textsuperscript{2+} channels are regulated differentially by distinct Ga subtypes. In order to unmask the effect of endogenous Ga, the antisense oligonucleotide, AGO, was routinely used in the following experiments using Xenopus oocytes (Figs. 2 and 3).

Effects of G\beta\gamma on the N- and P/Q-type Ca\textsuperscript{2+} Channels—Leu-EK-induced inhibitions of N-type, \(\alpha\text{1B} \) (Fig. 1A), and P/Q-type, \(\alpha\text{1A} \) (Fig. 1B), Ca\textsuperscript{2+} channels were not potentiated when G\(\beta\)\(\gamma\) was co-expressed. Alternatively, Ba\textsuperscript{2+} currents recorded from

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**FIG. 1.** Comparisons of the potencies of various G-protein subunit combinations in mediating agonist-induced inhibitions of \(\alpha\text{1B} \) and \(\alpha\text{1A} \) channels or native HVA Ca\textsuperscript{2+} channels. A and B, effects of Ga and G\(\beta\)\(\gamma\) on the agonist-induced inhibition of \(\alpha\text{1B} \) (A) or \(\alpha\text{1A} \) (B) currents without (upper) or with (lower) the injection of the antisense oligonucleotide, AGO, directed against mRNA encoding Xenopus Go\(\alpha\) (8). The receptor (DOR or \(\beta_{2}\)AR) and Ca\textsuperscript{2+} channel \(\alpha\text{1} (\alpha\text{1B or } \alpha\text{1A}), \alpha\text{2, and } \beta\text{1a subunits were co-expressed with Go or G\(\beta\)\(\gamma\) as indicated in Xenopus oocytes. In control oocytes, no exogenous Ga nor G\(\beta\)\(\gamma\) was expressed. When Go\(\alpha\) was co-expressed, \(\beta_{2}\)AR, instead of DOR, was expressed, and 1 \(\mu\)M isoproterenol, instead of 1 \(\mu\)M Leu-EK, was used for stimulating the receptor. In practice, the membrane was held at -80 mV and depolarized by a 250-ms test pulse from -80 mV to +10 mV. Since the peak current is inhibited prominently by Leu-EK (8), the amplitude of peak currents before and during exposure to Leu-EK or isoproterenol was used as a measure of the response to the agonist, and the change was expressed as a ratio of inhibition. Pretreatment (hatched bars) with 200 ng/ml PTX was carried out according to the procedures described under “Experimental Procedures.” The number of oocytes examined are indicated in parentheses. * and ** indicate significant differences (\(p < 0.05 \) and \(p < 0.01 \), respectively, by analysis of variance with post hoc test) when compared with controls for the three types of experiments such as antisense(/)/PTX(-), antisense(-)/PTX(+) and antisense(+). C, dose-dependent effects of Ga such as Go\(\alpha\) (open bars), G\(\beta_{3}\)\(\alpha\) (filled bars), and G\(\alpha\) (hatched bars) in potentiating Leu-EK-induced inhibition of \(\alpha\text{1B} \) currents. Ca\textsuperscript{2+} channel \(\alpha\text{1B}, \alpha\text{2, and } \beta\text{1a subunits cRNAs and DOR cRNA were co-injected with Ga cRNA at various concentrations indicated into Xenopus oocytes. The responses to 1 \(\mu\)M Leu-EK were measured and expressed as ratios of them to those in control oocytes, in which only Ca\textsuperscript{2+} channel subunits and DOR cRNAs were injected. The antisense oligonucleotide, AGO, was used. The number of oocytes examined are 8. The original responses for each before being normalized were 32.7 ± 3.5, 31.1 ± 4.2, and 25.8 ± 6.1%, respectively. D, potentiating effects of specific subtypes of Ga on ACh-induced inhibitions of HVA Ca\textsuperscript{2+} channels in NG108-15 cells. Current responses to a focal application of 1 mM ACh (3 \(\mu\)l) in untransfected NG108-15 cells (NG108) and their Go\(\alpha\)-transformed clones with (hatched bars) or without (open bars) pretreatment of 500 ng/ml PTX were expressed as ratios of inhibition. NG108-15 cells were transformed by Go\(\alpha\) as indicated. The number of oocytes examined are indicated in parentheses. * \(p < 0.05 \) when compared with control untransfected NG108-15 cells for the two types of experiment such as PTX(-) and PTX(+).
Fig. 2. Differential responses of α1B and α1A channels to the depolarizing prepulse in Xenopus oocytes co-expressed with Gβ1γ2 or Ga1, N-type (α1B, left), but not P/Q-type (α1A, right). Ca2+ channels in an oocyte implanted with DOR, Ca2+ channel α1 (α1a or α1A), α2 and β1a subunits, and Gβ1γ2 were prominently facilitated by a depolarizing prepulse (30 ms in duration) to +80 mV in the absence of Leu-EK. A 200-ms test pulse was applied to +10 mV from a holding potential of ~100 mV. When preceded by the conditioning depolarization, the test pulse was applied 20 ms after cessation of the prepulse. B, effects of changing duration of the prepulse on α1B (filled circles) and α1A currents (open circles) in oocytes expressed with DOR, Ca2+ channel α1 (α1a or α1A), α2 and β1a subunits, and Gβ1γ2. In practice, peak currents were measured before and after application of a prepulse to +80 mV, and the ratios were expressed as a function of the duration of the prepulse. The number of oocytes examined are 5. C, prepulse-resistant responses to Leu-EK in α1A channels. DOR and Ca2+ channel α1A, α2, and β1a subunits were co-expressed with Ga or Gβ1γ2 as indicated. In control oocytes (None), no exogenous Ga nor Gβ1γ2 was expressed. The responses of α1A currents to 1 μM Leu-EK (horizontal bars), prepulse (open circles), and both (filled circles) were measured and expressed as ratios. The same experimental protocols were used as in Fig. 1 for Leu-EK and as in Fig. 2A for prepulse. The number of oocytes examined for each data are 4-5. The antisense oligonucleotide, AGO, was used in A–C.

Oocytes expressed with the Ca2+ channel α1B, α2, and β1a subunits, DOR and Gβ1γ2 were facilitated by application of a large conditioning depolarization to +80 mV, in the absence of receptor stimulation (Fig. 2A, left). As mentioned before (8), this may indicate that the exogenous Gβγ can inhibit the N-type Ca2+ channel by itself, therefore without need for receptor-mediated activation of G-proteins. The prepulse facilitation was not prominent, but still significant, in the α1A channel (Fig. 2A, right).

Changing the prepulse duration more clearly revealed the difference in facilitation between α1B and α1A channels in oocytes co-expressed with Gβ1γ2 (Fig. 2B). A positive correlation between the extent of facilitation and prepulse duration was clearly observed in both α1B and α1A channels. However, the currents through α1A channels became suppressed as the duration of the prepulse was increased.

As shown in Fig. 2C (horizontal bar), Leu-EK-induced inhibition of α1A channel currents was markedly potentiated in oocytes co-expressed with G1a, G3a, or G5a, but this was not the case in oocytes co-expressed with Gβ1γ2. The prepulse procedure did not abolish the current inhibitions by Ga isoforms (filled circles), whereas it was abolished in α1B channels (Fig. 3A) as shown in the previous paper (8). Thus, the difference between α1B and α1A channels in G-protein modulation appeared to be more prominent for Ga than Gβ1γ2. Prepulse depolarization in the presence of Gβ1γ2 facilitated both α1B and α1A channels, but facilitation of currents that were suppressed by Ga was only observed for the α1B channel. These results suggest that Gαs are capable of distinguishing between HVA Ca2+ channel types.

Effects of Peptides Derived from Loop 1 and C terminus on the N- and P/Q-type Ca2+ Channels—Based upon evidence uncovering the structural determinants of Gaα and Gβγ interactions with α1B (8), an attempt was made to see whether
either the agonist-induced inhibition of a subunit co-expressed with G2b expressed as ratios. The pulse protocols were the same as those in Fig. 4. How-
terminus (see Fig. 4). The combination of PL1 and PB3T4, a derived peptide comprising a cysteine and the amino acid residues 2016–2025 or 1907–1925, respec-
tively (see Fig. 4), failed to suppress this inhibitory potentiation via Gβ1γ2 (relative response: 107.6 ± 0.6%, n = 4; also see Fig. 2, A and B) appeared to be inhibited by application of PL1 (98.2 ± 1.8%, n = 5).

In the case of α1b channels, the potentiating effects of Gα1o on the Leu-EK-induced inhibition of α1a channels (Fig. 3B, Gα1o, None, horizontal bar) were suppressed by the injection of the loop 1 peptide, PL1, together with one of the C-terminal peptides, PB3T1 or PPQT1 (Gα1o, PL1+PB3T1 and PL1+PPQT1, horizontal bars). The peptide PPQT1, an α1a version of PB3T4, was comprised of a cysteine and the amino acid residues 2028–2046 (Fig. 4). However, in contrast to α1b channels, PPQT1 by itself almost abolished the potentiating effects of Gα1o (Gα1o, PPQT1, horizontal bar), as observed with PL1 plus PPQT1. The amino acid sequences of α1g-derived PB3T1, PB3T2, PB3T3, and PB3T4 (other than the attached cysteine residue) share 60, 80, 13, and 53% identity with α1A, respectively (Fig. 4). PL1 alone, or combination of PL1 plus PB3T2 or PL1 plus PB3T3, did not reduce the potentiation of Leu-EK-induced current inhibition via Gα1o (Fig. 3B, Gα1o, PL1+PB3T2, and PL1+PB3T3, horizontal bars). Thus, the C-terminal peptide (PPQT1) was sufficient to impede the interaction of the P/Q-type α1A channel with Gα. On the other hand, the less prominent prepulse facilitation of the α1a channels via Gα1o was stimulated by Leu-EK in oocytes co-expressed with Gi3b, whereas that the loop 1 peptide alone was capable of impairing the interaction with Gβγ.

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Gβ purified from brain (Fig. 5B, lanes 1, 3, 5 and 7), as well as a 36-kDa polypeptide released from the GST-B3L1-bound beads (Fig. 5B, lane 4, arrowhead) that had been incubated with the purified Gβγ2. Both reactivities of M-14 with the 36-kDa polypeptides were inhibited by preincubation of M-14 with the peptide antigen sc-261P for M-14 (n = 3). No 36-kDa polypeptide, however, was detected by M-14 in polypeptides released from GST-B3T-bound beads despite the incubation with the purified Gβγ2 (Fig. 5B, lane 2, arrowhead). These results support the idea that Gα and Gβγ inhibit the N-type Ca2+ channel by directly interacting with the C terminus and the loop 1 of the channel, respectively.

**DISCUSSION**

In the present study, we found that distinct sets of Gα co-expressed in oocytes mediated receptor agonist-induced inhibitions of N-type α1B and P/Q-type α1A channels; agonist-induced inhibition of the α1B channel was potentiated by co-expression of Gi3α, Gγ1α, Gγ3α, and Gα, whereas that of the α1A channel was intensified by co-expression of Gγ1α, Gγ2α, Gγ3α, or Gα. Single channel recordings indicated that the molecular species Gi3α is a PTX-sensitive G-protein in native tissues and...
Finally, we defined the loop 1 of a presence (washing, proteins bound to beads were released by boiling and separated by 11% SDS-PAGE, together with 25 ng (A, lanes 1, 3, 5, and 7) or 5 ng (A, lanes 9 and 11) of purified Gα, or 25 ng of Gβγ3 (B, lanes 1, 3, 5, and 7). Immunoblot analysis was performed, as described under “Experimental Procedures,” using the antibodies Gα AB (A, lanes 1-6) and K-20 (A, lanes 9-12) against Gα, and the antibody M-14 against Gβ (B). The incubation with the antibody GAB was carried out in the presence (A, lanes 3, 4, 7, and 8) or absence (A, lanes 1, 2, 5, and 6) of 0.1 mg/ml of the peptide antigen FG, or GAB. In the case of K-20, the antibody was preincubated with ∆A, lanes 11 and 12) or without (A, lanes 9 and 10) the peptide antigen sc-387P for K-20 according to the procedure described by the vendor. The first antibodies described above were diluted at 1:2000 (B, lanes 1 and 2), 1:4000 (A, lanes 1-8; B, lanes 3 and 4), or 1:8000 (B, lanes 9-12; B, lanes 5-8). Arrowheads indicate the positions of purified Gα (A) and Gβ (B). The size markers used were the Wide Range SigmaMarkers (Sigma). Note that the 38-14A purified Gα (A, lane 5) is in a higher position than the major nonspecific bands (A, lane 6), which were not inhibited by the peptide antigen (A, lane 8).

elicits channel inhibition through a membrane-delimited pathway (33, 34). Alternatively, a depolarizing prepulse relieved channel inhibition caused by the Gβγ complex, with facilitation being more pronounced in α1B than in α1A channels. Finally, we defined the loop 1 of α1B and α1A as an interaction site for Gβγ and the C terminus of α1B and α1A for Gα, based on the direct binding of Gα and Gβγ2 in vitro to channel segments, as well as the responses of wild-type channels to synthetic peptides. Gα, but not the Gβγ complex, purified from bovine brain bound in vitro to the C terminus of α1B and α1A channels, which was fused as a GST protein. Conversely, Gβγ2 bound in vitro to the loop 1 of α1B and α1A channels as described (9, 10). The obtained results provide evidence that Gα as well as Gβγ directly interact with Cα2+-channel α1 subunits to inhibit their activity.

Differential Regulation of α1A and α1A Channels by Gα and Gβγ—Gα, Gα (1, 2, 4, 6) and, more recently, Gβγ (35, 36) have been shown to be involved in inhibitory modulation of HVA Ca2+-channels, including N-type Cα2+-channels. Among the six different subtypes of Gα examined, particular subtypes (Gα3, Gα, Gα, and Gα) further intensified the agonist-induced inhibition of α1B channels, whereas Gα3 and Gα did not, despite the fact that they are able to couple to DOR (37, 38). Therefore, these Gα subtypes seem to be unable to inhibit the α1B channel. Moreover, it seems unlikely that the inhibitory action of Gα is associated with adenylyl cyclase, since neither intracellular application of cyclic AMP nor a catalytic subunit of PKA nor pretreatment with H7 altered the α1B channel activities. Our findings of Gα-induced inhibition of N-type Cα2+-channels are consistent with evidence that inhibition of N-type Cα2+-channels by vasoactive intestinal polypeptide is attenuated by cholera toxin and anti-Gα antibodies (39). Similar preferences among Gα subtypes in potentiating ACh-induced inhibition were observed in NG108-15 cells, in which N-type, but not P/Q-type, Cα2+-channels were natively expressed. Inhibition of α1A channels by Leu-EK was potentiated when Gα, Gα, Gα, or Gα was co-expressed. Thus, the α1B and α1A channels presumably carry interaction sites that are capable of selectively recognizing certain subtypes of G-protein α subunits.

When Gα cRNA or a low concentration of Gα cRNA was co-injected, the Leu-EK-induced inhibition of α1A channels was considerably reduced. The potency of Gα in inhibiting α1B channels was lower than those of Gα, Gα, and Gα. This attenuation of the channel inhibition by Gα, Gα, and Gα may be due to a trap of the endogenous Gβγ by the exogenous Gα, leading to occlusion of an inhibitory signal by Gβγ. Our previous studies have suggested the presence of blockade by exogenous Gα in the metabolotropic glutamate receptor-induced phosphoinositide hydrolysis (12).

When Gβγ was co-expressed, the Leu-EK-induced inhibition was not potentiated in either α1B or α1A channels. In the case of α1B, however, a depolarizing prepulse to +80 mV facilitated the currents in the absence of the receptor agonist, suggesting that the exogenous Gβγ inhibits the α1A channel by itself (35, 36). As shown in the previous paper (8), the prepulse did not affect α1B channels in oocytes co-expressed with Gβγ unless DOR was stimulated by Leu-EK. Thus, it appears that the exogenous Gα does not affect the α1B channel by itself and that the channel inhibition observed with the exogenous Gα results from interaction of the channel with the exogenous Gα and/or an endogenous Gβγ released from the exogenous Gα.

In the case of the α1A channel, the prepulse facilitation was not prominent when Gβγ was co-expressed. Furthermore, as the duration of prepulse was increased from 30 to 50 ms, the facilitation practically disappeared in α1A channel, whereas it remained unchanged in α1B channel, probably reflecting the difference in voltage-dependent channel inactivation (16). On the other hand, Leu-EK-induced inhibition of the α1A channel was markedly potentiated in oocytes co-expressed with Gα subtypes, similar to the α1B channel. The prepulse failed to abolish this inhibition potentiated by Gα subtypes in α1A but not in α1B channels (8).

All these findings indicate that the N- and P/Q-type Cα2+-channels are regulated differentially by distinct Gα subtypes and that the N-type is preferentially regulated by the Gβγ subunit. In addition, it has been suggested that in N- and P/Q-type Cα2+-channels, there is a structural domain associated with G-proteins, which has a voltage sensitivity distinguishable by prepulse (40).
indicating that there is an additional interaction site for G-protein subunits outside of loop 1 (40). The same conclusions were drawn by using channel mutation and chimerization in the previous study (8).

The Gαq-dependent potentiation in α₁B channels was blocked by co-application of the peptide PL1 and an α₁G C-terminal peptide (PB3T1 or PB3T4) but not blocked by the C-terminal peptide alone. These results indicate that both loop 1 and C-terminal segment of α₁B play an essential role for the Gαq-dependent potentiation and that the interaction site for Gαq seems to be mainly assigned to the α₁B C terminus.

In the P/Q-type α₁A channel, an α₁A version of the C-terminal peptide PB3T4 (PPQT1), was able to diminish the potentiation of agonist-induced current inhibition via Gqα without the aid of the loop 1 peptide PL1. The results indicate, as in the case of α₁B, that the C-terminal segment of α₁A is essential for the interaction with G-protein subunits, whereas the loop 1 of α₁A is not essential.

Direct Binding of Ga with the C Terminus of α₁B and α₁A Channels, and Gβγ by the Loop 1 of the Channels—Finally, we found that bacterial fusion proteins containing the C-terminal segment of the N- (α₁B) and P/Q-type (α₁A) Ca²⁺ channels were capable of binding bovine brain purified Gα but not Gβγ. In addition, GST fusion proteins containing the loop 1 of α₁B and α₁A were able to bind the Gβγ but not the Gαq as reported recently (9, 10). It has been shown more recently that Gβγ also binds to the C terminus of the α₁B channel in vitro (41). This result suggests that each type of neuronal Ca²⁺ channel is differentially regulated by each subunit of the G-protein complex as observed with the α₁B and α₁A channels, in which contribution of the α₁A loop 1 to channel modulation by Ga was smaller than that of the α₁B loop 1. In addition, the C-terminal short fragments of α₁B and α₁A have been shown to be bound by Gβγ (41). The disparity may imply the presence of an additional C-terminal domain that affects the interaction with Ga and Gβγ. This speculation is consistent with the fact that the corresponding positions of the C-terminal peptides (PB3T4 and PPQT1) on α₁B and α₁A are different from those of the Gβγ-bound fragments reported. Further studies using mutagenesis will be necessary to identify the specific amino acid residues on α₁B and α₁A determining the interactions with Ga and/or Gβγ, or determining the differences in modulatory properties between N- and P/Q-type Ca²⁺ channels.

All of these findings from the present and the previous experiments (8) indicate that the C terminus of N- and P/Q-type Ca²⁺ channels contain an interaction site for Gα and that the loop 1 contains an interaction site for Gβγ. It is further indicated that the Ga species Gα₁A and Gα₁B are shared by the N- and P/Q-type Ca²⁺ channels, whereas Gα₂ and Gα₄ are rather specialized for inhibiting the N-type and Gα₁C and Gα₂βγ for selectively suppressing the P/Q-type. Since multiple G-protein isoforms co-exist with more than two types of HVA Ca²⁺ channels in a single neuronal cell, switching on/off the expression of a particular Ga subtype would convert either one or both of the N- and P/Q-type channels to a sensitive or insensitive response to an inhibitory signal by the same transmitter/agonist stimulation. Thus, the regulation of the N-type and P/Q-type Ca²⁺ channels by different Ga and Gβγ would allow a variability and a flexibility in synaptic efficacy by alteration of transmitter release.

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