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Direct interaction of Gβγ with a C-terminal Gβγ-binding domain of the Ca^{2+} channel α_1 subunit is responsible for channel inhibition by G protein-coupled receptors

(synaptic transmission/protein–protein interaction/signal transduction/calcium/neurosecretion)

NING QIN*†, DANIELA PLATANO*†, RICCARDO OLCESE*, ENRICO STEFANI*‡§, AND LUTZ BIRNBAUMER*‡§¶‖

Departments of *Anesthesiology, ¶Biological Chemistry, and ‡Physiology, and the §Brain Research and ‖Molecular Biology Research Institutes, University of California, Los Angeles, CA 90095

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ABSTRACT **Several classes of voltage-gated Ca^{2+} channels (VGCCs) are inhibited by G proteins activated by receptors for neurotransmitters and neuromodulatory peptides. Evidence has accumulated to indicate that for non-L-type Ca^{2+} channels the executing arm of the activated G protein is its βγ dimer (Gβγ). We report below the existence of two Gβγ-binding sites on the A-, B-, and E-type α_1 subunits that form non-L-type Ca^{2+} channels. One, reported previously, is in loop 1 connecting transmembrane domains I and II. The second is located approximately in the middle of the *ca.* 600-aa-long C-terminal tails. Both Gβγ-binding regions also bind the Ca^{2+} channel β subunit (CCβ), which, when overexpressed, interferes with inhibition by activated G proteins. Replacement in α_{1E} of loop 1 with that of the G protein-insensitive and Gβγ-binding-negative loop 1 of α_{1C} did not abolish inhibition by G proteins, but the exchange of the α_{1E} C terminus with that of α_{1C} did. This and properties of α_{1E} C-terminal truncations indicated that the Gβγ-binding site mediating the inhibition of Ca^{2+} channel activity is the one in the C terminus. Binding of Gβγ to this site was inhibited by an α_1-binding domain of CCβ, thus providing an explanation for the functional antagonism existing between CCβ and G protein inhibition. The data do not support proposals that Gβγ inhibits α_1 function by interacting with the site located in the loop I–II linker. These results define the molecular mechanism by which presynaptic G protein-coupled receptors inhibit neurotransmission.**

Studies on stimulation-evoked release of norepinephrine from sympathetic terminals of the cat's nictitating membrane before and after α-adrenergic blockade led to the discovery in 1971 of an inhibitory presynaptic α adrenoreceptor, now known as one of the α_2-adrenoreceptors (1). Presynaptic inhibition of neurosecretion by the released neurotransmitter (2) or by neuropeptides (3), all acting through G protein-coupled receptors, is now recognized as an important regulatory feedback mechanism utilized throughout the central and the peripheral nervous system. Evidence has accumulated to indicate that this type of inhibition of neurotransmitter release is due to inhibition of presynaptic N- and P/Q-type Ca^{2+} channels (4–9) by a mechanism that is likely to use the βγ signaling arm of activated G proteins (10, 11).

Voltage-gated Ca^{2+} channels are multisubunit complexes formed of a pore-forming and voltage-sensing α_1 subunit, a regulatory $\alpha_2\delta$, and one or possibly two (12) regulatory β subunits. Voltage dependence; fundamental aspects of activation, deactivation, and inactivation; feedback inhibition by Ca^{2+}; and sensitivity to various Ca^{2+} channel blockers are all encoded in α_1 subunits, of which there are six major types (S, A, B, C, D, and E). Each is subject to modulation to variable degrees by the named regulatory subunits, and each is expressed in alternatively spliced forms (reviewed in refs. 13–15). The N- and P/Q-type Ca^{2+} channels regulated negatively by a G protein-coupled pathway involving Gβγ are encoded in the A-, B-, and E-type α_1 subunits (reviewed in ref. 16). Studies carried out primarily with endocrine cells (refs. 17 and 18; reviewed in ref. 19) and, more recently with cardiomyocytes derived from a $G_o\alpha$ knockout mouse (20), have shown that at least one subtype of L-type Ca^{2+} channels is also subject to inhibitory regulation by a G protein-coupled pathway. In contrast to the regulation of non-L-type Ca^{2+} channels by a membrane-delimited pathway, regulation of L-type Ca^{2+} channels by a G protein-coupled pathway is thought to depend on the intermediary activation of a phosphoprotein phosphatase and appears therefore to involve phosphorylation/dephosphorylation cycle, affecting an as yet unidentified component of the Ca^{2+} channel (20, 21).

In addition to the "primary" regulation by β, $\alpha_2\delta$ and an activated G protein, N- and/or P/Q-type channels are further fine-tuned by a cross-talk between calcium channel β subunits (CCβs) and activated G proteins. This was shown by Dolphin and collaborators (22), who found that inhibition of Ca^{2+} channel currents in dorsal root ganglion cells by the $GABA_B$ agonist baclofen is enhanced in cells in which CCβ subunits had been depleted by previous injection of specific antisense oligonucleotides. This led them to propose that CCβ subunits attenuate or antagonize inhibitory regulation by G proteins.

The inhibitory regulation of voltage-gated Ca^{2+} channels by G protein activation seen in neurons and neuronal-type cells (refs. 4–9; for review see ref. 16) has been reconstituted by expression of cloned α_1 subunits in *Xenopus* oocytes (23) and in mammalian cells (11). Likewise, the antagonism discovered by Dolphin and collaborators (22) between CCβ and inhibition by G protein activation (24, 25) has also been reconstituted in *Xenopus* oocytes, as G protein activation fails to inhibit α_{1A} currents in oocytes coexpressing a CCβ (24, 25). These findings opened the possibility to answer questions as what is the real molecular (subunit) nature of the regulated channels, whether activated G proteins interact directly with one of the components of the Ca^{2+} channel complex, and, if so, with which, and whether β subunits and activated G proteins interact competitively.

In support of the proposal of Ikeda (10) and Herlitze *et al.* (11) that Gβγ may be acting by binding directly to one of the components of the non-L-type α_1 subunits, Zamponi *et al.* (26) and De Waard *et al.* (27) discovered the existence of a

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Abbreviations: CCβ, calcium channel β subunit; CCh, carbachol; Gβγ, G protein βγ dimer; GST, glutathione *S*-transferase; M2R, type-2 muscarinic acetylcholine receptor; VGCC, voltage-gated calcium channel.

†N.Q. and D.P. contributed equally to this work.

Gβγ-binding activity in G protein-sensitive α_{1A} and α_{1B} subunits that is absent in the G protein-insensitive α_{1C}. This binding activity is located in the cytosolic loop that connects the homologous hydrophobic repeat domains I and II (loop 1), which incidentally also contains the primary CC β-binding site (28). De Waard *et al.* (27) reported further that a mutation of α_{1A}, R387E, that interferes *in vitro* with Gβγ binding interfered in *Xenopus* oocytes with development of the inhibition by activated G protein, and they proposed that Gβγ acted to inhibit Ca^{2+} channel activity by binding to the loop 1 site identified through the *in vitro* binding studies. However, Zhang *et al.* (29) reported that an α_{1B}/α_{1C} chimera that should not have bound Gβγ exhibits a normal inhibitory response to G protein activation, and, more recently, Herlitze *et al.* (30) reported that α_{1A}[R387E] expressed in HEK cells is inhibited by activated G protein. It thus appears questionable whether the Gβγ-binding site discovered by Zamponi *et al.* (26) and De Waard *et al.* (27) is indeed relevant to G protein-induced inhibition of neuronal Ca^{2+} currents, and, by extension, it remains to be determined whether α_1 is indeed the direct target (effector) of the activated G proteins.

We have also searched for a Gβγ-binding site on an α_1 subunit, but instead of working with the α_{1A} or α_{1B} subunit, we worked with α_{1E}, which, like α_{1A} and α_{1B}, is also subject to negative regulation by G protein-coupled receptors (31). We found that Gβγ interacts with two α_{1E} sites, which, coincidentally, colocalize with the two recently identified CC β-binding sites (12): one is located in the loop connecting homologous repeat domains I and II (28), and the other is in the C-terminal tail. In contrast to the proposals of Zamponi *et al.* (26) and De Waard *et al.* (27), we find no evidence that the loop 1 site is involved in mediation of the inhibitory effect of activated G proteins. In contrast, all our data point to the C-terminal Gβγ-binding site as the one that mediates the action of Gβγ. *In vitro* binding of Gβγ to the C-terminal site is prevented by coincubation with a recombinant α_1-binding CCβ fragment.

METHODS

Glutathione *S*-Transferase-α_{1E} Fusion Proteins. GST-α_{1E} fusion plasmids were based on pGEX-4T-1 (Pharmacia) and were constructed by conventional means using either natural restriction fragments of α_1 subunits or defined fragments excised by PCR. After transfection into *E. coli* BL21, synthesis of the fusion proteins was induced with 0.2 mM isopropylthiogalactoside (IPTG) in a liquid culture grown to OD of 1.0. After 2–3 hr at 37°C the cells were collected by centrifugation, resuspended in NETN lysis buffer (1.0 ml per 20 ml culture; NETN, 0.5% Nonidet P-40/1 mM EDTA/20 mM Tris·HCl, pH 8.0/100 mM NaCl) and lysed by sonication. The lysate was cleared by centrifugation at 10,000 × *g* for 10 min at 4°C. The GST-fusion proteins in the supernatant were adsorbed to glutathione (GSH)–Agarose beads for 30 min at room temperature in NETN (1 vol lysate: 1 vol 50% (vol/vol) slurry of Agarose–GSH beads (Pharmacia) in NETN). The last wash was with binding buffer [1% vol/vol Lubrol-PX (Sigma)/2 mM EDTA/100 mM NaCl/20 mM Tris·HCl, pH 8.0] instead of NETN.

G protein βγ dimers were purified from human or porcine erythrocyte membranes (32) and from bovine brain (33). Bovine brain G_o was purified as described previously (34). ^{35}S-labeled forms of rat β2a (35), β2a[D1–3] (β2a[1–211]), and β2a[D4] (β2a[206–415]) and β2a[D4–5] (β2a[206–604]) were synthesized in rabbit reticulocyte lysates by coupled transcription–translation (Promega) in the presence of [^{35}S]methionine as described by Pragnell *et al.* (28). β2a[D1–3] and β2a[D4], each with a hexa-histidine tag at its N terminus, were synthesized in *Escherichia coli* (strain BL21[DE3]) fused to thioredoxin using the pET-32a(+) vector and reagents supplied in kit form by Novagen. Single colonies of transformed cells were expanded, inoculated into 100 ml of Luria–Bertani medium, grown to OD 1.0, induced with 0.4 mM IPTG for 2 h at 37°C, and harvested by centrifugation. The pellet was resuspended in 10 ml of 5 mM imidazole/500 mM NaCl/20 mM Tris·HCl, pH 7.9 (buffer A), sonicated, and centrifuged in the cold at 27,000 × *g* for 15 min. β2a[D1–3] was purified from the supernatant by Ni affinity chromatography (1-ml bed volume), followed by dialysis against 100 mM NaCl/2 mM EDTA/20 mM Tris·HCl, pH 7.5 (buffer B). β2a[D4] was solubilized from the pellet with 10 ml of 6 M urea in buffer A (1 h at 4°C), followed by centrifugation as above. The supernatant was diluted with 1 vol of buffer A, and the protein was adsorbed onto immobilized Ni (1-ml bed volume equilibrated in buffer A containing 2 M urea). After washing the resin with 2 M urea in buffer A, the protein was eluted with 5 ml of 1 M imidazole/0.5 M NaCl/Tris·HCl, pH 7.5, containing 2 M urea. The eluate was dialyzed against buffer B with decreasing concentrations of urea, ending with an overnight dialysis against buffer B without urea, all at 4°C.

Protein–Protein Interactions. Twenty-five percent (vol/vol) slurries of Agarose-GSH beads with approximately 1 μg of GST or GST-α_1[frg] were incubated for 30 min at room temperature in a final volume of 100 μl of binding buffer (buffer B plus 1% Lubrol-PX) without or with 1 μg (20 pmol) Gβγ, 10 μl reticulocyte lysate containing 10–30 nM ^{35}S-labeled β2a fragments, or 100–500 pmol thioredoxin-His_6-β2a.frg. At the end of the incubations the beads were washed three times with 1.0 ml of binding buffer and resuspended in 15 μl of Laemmli's 2× sample buffer. Proteins released from the beads were analyzed by 10% SDS/PAGE followed by autoradiography to detect binding of [^{35}S]β2a fragments or by Western blotting to determine binding of G protein β subunits using rabbit anti-β_{common} antibodies (gifts from Suzanne Mumby and Alfred Gilman, University of Texas, Dallas, and from Guenter Schultz, University of Berlin). Rabbit IgG was revealed by ECL (Amersham).

α_1 Subunit Constructs and Synthesis of cRNAs. α_1 cDNAs were wild-type (wt) α_{1E}, α_{1E}[1–2312], clone 239 of Schneider *et al.* (36); α_{1C}[DN60], α1C[60–2171] (37), α_{1E}[DC277], α_{1E}[1–2035]; α_{1E}[DC244], α_{1E}[1–2068]; chimera EC1 (α_{1E} with α_{1C} C-terminal tail): α_{1E}[1–1728]/α_{1C}[1513–2171]; chimera EC30 (α_{1E} with L1 of α_{1C}): α_{1E}[1–337]/α_{1C}[421–583]/α_{1E}[503–2312]. Deletion mutants and chimeras were made by standard recombinant DNA techniques using wild-type α_{1E} and α_{1C}[DN60] cDNAs as donor DNAs. All cDNAs were subcloned into the *Nco*I site of the transcription competent pAGA2 plasmid (38, 39). cRNAs were synthesized using mMessage mMachine reagents and protocols purchased in kit form from Ambion (Austin, TX). The resulting cRNAs were resuspended in diethylpyrocarbonate-treated H_2O.

***Xenopus* Oocytes, Expression of Calcium Channels, and Electrophysiological Recordings.** Stage V and VI *Xenopus laevis* oocytes, isolated as described in Tareilus *et al.* (12) and injected with 50 nl containing 100 μg/ml each of two cRNAs: one encoding one of the α_1 subunits and the other encoding the human type-2 muscarinic acetylcholine receptor (40), also transcribed from pAGA2. The cut-open vaseline gap voltage-clamp method of Taglialatela *et al.* (41), as modified (42, 43), was used throughout. The external solution had the following composition: 10 mM Ba^{2+}/96 mM Na^+/10 mM Hepes, titrated to pH 7.0 with methanesulfonic acid (CH_3SO_3H). The solution in contact with the oocyte interior was 110 mM K-glutamate/10 mM Hepes, titrated to pH 7.0 with KOH. Low-access resistance to the oocyte interior was obtained by permeabilizing the oocyte with 0.1% saponin. For further details see Noceti *et al.* (43). Currents were recorded 3–5 days after cRNA injection. Test protocols are depicted on the figures.

RESULTS

Fig. 1 shows the results from experiments in which we tested the ability of various fragments of the neuronal, G protein-sensitive α_{1E} fused to GST and immobilized on glutathione-Agarose for their ability to bind purified bovine brain G protein $\beta\gamma$ dimers. Of the regions tested, we found two that bound G$\beta\gamma$ with sufficient avidity to withstand washing: the loop connecting repeat domains I and II (L1) and the carboxyterminal half of the C-terminal tail (Fig. 1*B*). In agreement with the findings of Zamponi *et al.* (26) and DeWaard *et al.* (27), G$\beta\gamma$ bound also to the L1 regions of the G protein-sensitive α_{1A} and α_{1B} (not shown). Successively smaller fragments of the α_{1E} C-terminal tail showed that the G$\beta\gamma$-binding activity resides in fragment CC14, a 38-amino acid stretch located approximately in the middle of the tail (Fig. 1*C*). The need for free G$\beta\gamma$ was tested by incubating α_{1E}[L1] and α_{1E}[CC14] with unactivated bovine brain G_o before and after its activation by GTP[γS]. G$\beta\gamma$ in the heterotrimeric G_o did not bind to α_{1E} fragments, but the G$\beta\gamma$ released from a G_o by treatment with 100 μM GTS[γS] and 10 mM $MgCl_2$ did (Fig. 1*D*). In other experiments we found that the α_{1E}[CC14] region recognizes not only the bovine brain G$\beta\gamma$ but also G$\beta\gamma$ purified from human and porcine erythrocytes (data not shown).

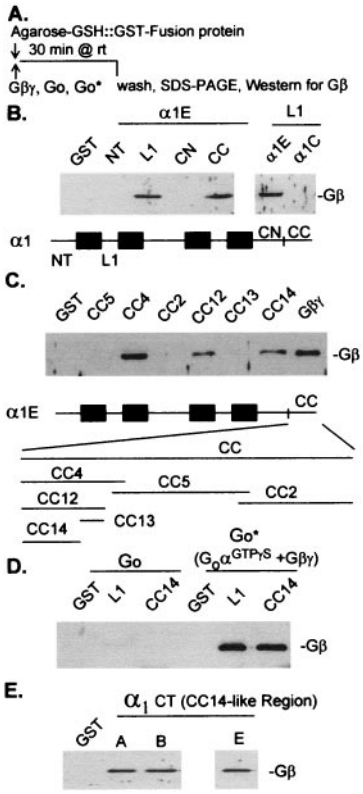

FIG. 1. Interaction between fragments of α_{1E} and G$\beta\gamma$. Binding of G$\beta\gamma$ to fragments of α_1 fused to GST was analyzed by Western blotting with an anti-$G\beta_{common}$ antibody. The figure shows digitized pictures of autoradiograms identifying the 35-kDa Gβ subunit. Here and throughout, α_1 subunits are represented as homologous hyrophobic repeat domains I–IV (boxes) connected by cytosolic loops (L1 through L3) with N-terminal (NT) and C-terminal extensions. CN, N-terminal portion of a C terminus; CC, C-terminal portion of a C terminus. α_{1E} is represented by black repeat domains connected by heavy lines denoting N and C termini and the connecting loops; α_{1C} is represented by open boxes connected by thin loops and flanked by thin N and C termini. (*A*) Outline of the experimental protocol. (*B* and *C*) Binding of G$\beta\gamma$ to α_1 fragments. (*B*) NT, α_{1E}[1–89]; L1, α_{1E}[356–451]; CN, α_{1E}[1712–1980]; CC, α_{1E}[2036–2312]; α_{1C} L1, α_{1C}[436–554]. (*C*) Fragments of the CC region of α_{1E}: CC4, α_{1E}[2036–2136]; CC5, α_{1E}[2122–2240]; CC2, α_{1E}[2220–2312]; CC12, α_{1E}[2036–2093]; CC13, α_{1E}[2075–2093]; CC14, α_{1E}[2036–2074]. G$\beta\gamma$, 12% SDS/PAGE and Western blot of 100 ng bovine brain G$\beta\gamma$. (*D*) Only free G$\beta\gamma$ interacts with L1 and CC14. G_o, 200 nM purified bovine brain G_o in binding buffer (see *Methods*); G_o*, 200 nM G_o after 30-min treatment at 32°C with 100 μM GTP[γS] and 10 mM $MgCl_2$ in binding buffer. (*E*) Binding of G$\beta\gamma$ to C-terminal fragments of α_{1A} and α_{1B} (α_1CT fragments). α_1A CT, α_{1A}[[2150–2216]; α_{1B} CT, α_{1B}[2013–2069]; α_{1E} CT = CC4 or α_{1E}[2036–2136]. All α_1 fragments were fused to GST. α_1 numbers correspond to the amino acids of the respective α_1 subunits that make up the fragments fused to GST; numbering is according to GenBank L277450 for α_{1E}, GenBank X15539 for α_{1C}, GenBank X57476 for α_{1A}, and GenBank U04999 for α_{1B}. β2a (rat) is numbered according to GenBank M80545. In this and the other figures GST denotes incubation of G$\beta\gamma$ or [^{35}S]β2a with Agarose-GSH::GST without α_1 fragments fused to the GST. CC14 or α_{1E}[2036–2073] = MERSSENTYK ARRRSYHSSL RLSAHRLNSD SGHKSDTH.

The discovery that α_{1E} has two G$\beta\gamma$-binding sites required that we search for a functional correlate that would indicate whether one, both, or neither of these sites is involved in inhibitory regulation of neuronal Ca^{2+} channels. To this end, α_{1E} Ca^{2+} channels were expressed in *Xenopus* oocytes together with the M2 muscarinic receptor (M2R), which is coupled to effector functions by the G_i/G_o group of G proteins, and analyzed the inhibition of Ca^{2+} channel currents by the muscarinic agonist carbachol (CCh). Lux and coworkers (44, 45) and Pollo *et al.* (46) showed that inhibition by G protein-coupled receptors is relieved by strong depolarizations, a phenomenon that has since been recapitulated in many other studies (e.g., ref. 9), including those of Ikeda (10) and Herlitze *et al.* (11), which point to G$\beta\gamma$ as the executing arm of activated G proteins. We thus tested for reconstitution of the G protein-dependent regulation in the oocyte both by eliciting the agonist-mediated reduction in current amplitude and/or by assessing the concurrent appearance of its reversal by a depolarizing prepulse.

Fig. 2 illustrates the characteristics of inhibition of α_{1E} currents triggered by M2R in Xenopus oocytes and the lack of

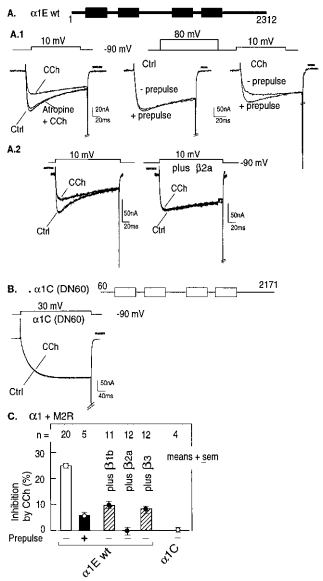

FIG. 2. Regulation of α_{1E} but not α_{1C} by a G_i/G_o-coupled receptor; reversal of α_{1E} inhibition by ligand antagonist and depolarizing prepulse and prevention by coexpression of calcium channel β2a subunit. All oocytes were injected with M2R and the indicated α_1 and β subunits. (*A* and *B*) Representative records. Test protocols are shown above the current traces. (*C*) Summary of results. CCh was 50 μM, atropine (in the presence of CCh, was 0.5 μM. Inhibition by CCh (%) = I_{Ba} after 50 μM CCh/I_{Ba} after CCh washout $\times$ 100. I_{Ba} were measured isochronically at the peak of the control current after CCh washout. The bars represent means ± SEM of the indicated number of oocytes.

an effect on α_{1C}. As seen in 20 oocytes, activation of M2R with CCh reduced peak currents 25.1 ± 0.6% (mean ± SEM; Fig. 2 *A* and *C*). This inhibition was reversed by the muscarinic receptor antagonist atropine (Fig. 2*A Left*) and by depolarizing prepulses (Fig. 2*A.1* and *C Center* and *Right*). Coexpression of β2a interfered with muscarinic inhibition of α_{1E} (Fig. 2*A.2*), and the degree of inhibition was dependent on the type of CCβ tested: β2a essentially abolished the effect of M2R, whereas β1b and β3 inhibited it by only about 50–60% (Fig. 2*C*). In contrast to α_{1E}, and in agreement with previous studies (10), α_{1C} channels failed to be inhibited by activation of a G_i/G_o-coupled receptor (Fig. 2 *B* and *C*).

To determine which of the two Gβγ-binding sites had the potential of mediating inhibition of α_{1E} currents, we tested two α_{1C}/α_{1E} chimeras. In EC30 we replaced the α_{1E} L1 segment with that of α_{1C}, which is unable to bind Gβγ (Fig. 1). In EC1 we replaced the complete C terminus of α_{1E} with that of the G protein-insensitive α_{1C}. As shown in Fig. 3, the regulation by M2R was lost in EC1, whereas it was retained in EC30. These results indicated that of the two Gβγ-binding sites discovered in the experiments of Fig. 1, only the one located in the C terminus could be of importance and that Gβγ binding to the L1 segment was not involved in the inhibitory regulation of these channels.

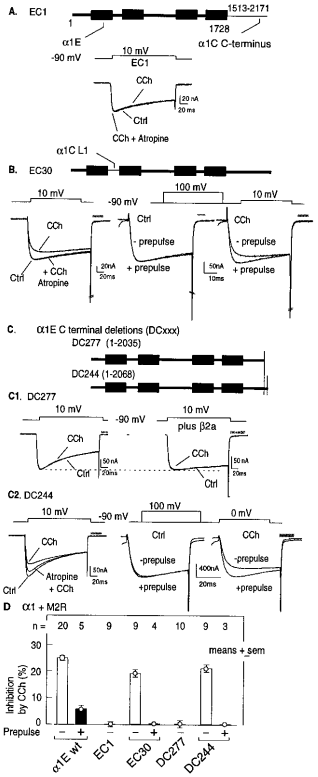

FIG. 3. Functional identification of a 33-aa region of α_{1E} that confers susceptibility to regulation by a G protein-coupled receptor. All oocytes were injected with M2R and the indicated α_1 cRNAs. (*A*) Lack of inhibition by M2R of EC1, a chimera formed of α_{1E} with an α_{1C} C terminus. (*B*) EC30, a chimera formed of α_{1E} with an α_{1C} L1 region, is susceptible to inhibition by M2R, and this inhibition is sensitive to a depolarizing prepulse. (*C*) Truncation of the C-terminal tail of α_{1E} by 277 amino acids, α_{1E}[1–2035] (DC277 on figure), eliminates the effect of CCh (*C1*), but removal of 244 amino acids, α_{1E}[1–2068] (DC 244 on figure), does not eliminate inhibitory regulation by the G protein-coupled receptor, seen as CCh-induced reduction in activity that can be blocked either by atropine or by a depolarizing prepulse (*C2*). Note that the effect of β2a to slow the rate of α_{1E} inactivation is still present in DC277. (*D*) Summary of effects of M2R activation on α_{1E}/α_{1C} chimeras and C-terminally truncated, mutated α_{1E} constructs. α_{1E} wild-type data are the same as in Fig. 1*B*.

Two α_{1E} mutants showed that the C-terminal Gβγ-binding site is essential for responsiveness to G protein activation (Fig. 3 *C* and *D*). The inhibitory response to G protein activation was retained in α_{1E}[DC244], an α_{1E} that lacks its last 244 amino acids but retains 35 of the 38 amino acids that constitute the Gβγ-binding α_{1E}[CC14] characterized in Fig. 1, whereas it was lost in α_{1E}[DC277], an α_{1E} that is truncated just prior to the beginning of the Gβγ-binding fragment (Fig. 3*C Center* and *Right*). In contrast to the loss of inhibitory regulation by M2R, α_{1E}[DC277] retained full sensitivity to regulation by β2a. This was assessed by expressing α_{1E}[DC277] alone and in combination with β2a. β2a caused (*i*) a slowing of the rate of inhibition by voltage (Fig. 3*C*), (*ii*) a shift in the voltage dependence for activation (data not shown), and (*iii*) a shift in the midpotential of inactivation (data not shown), as it does when expressed with the wild-type α_{1E} (47).

Amino acid alignments showed that the C-terminal tails of α_{1B} and α_{1A}, but not the tail of α_{1C}, contain a sequence that is

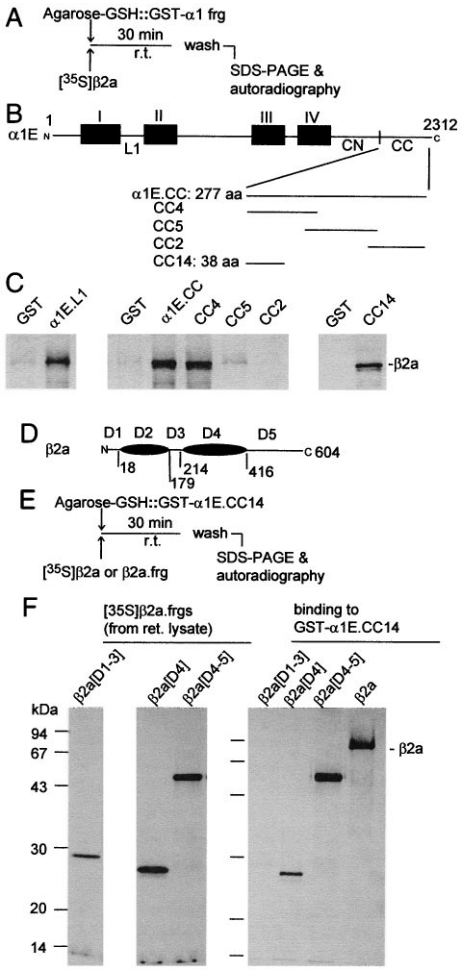

FIG. 4. CC14 (α_{1E}[2036–2074]), the C-terminal Gβγ-binding domain of α_{1E}, is also a CCβ-binding domain, and CCβ2a[206–412] contains the α_1-binding domain of CCβ2a. (*A–C*) Localization of a β2a-binding site within the α_{1E} C terminus. (*A*) Outline of experiment. (*B*) Ideogram of α_{1E} and α_{1E} fragments tested as GST fusions for β2a-binding activity. (*C*) Binding of [^{35}S]β2a to the fragments shown in *B*. CC, CC2, CC4, CC5, and CC14 are the same as in Fig. 1. (*D–F*) Binding of β2a fragments to α_{1E}[CC14]. (*D*) Ideogram of β2a. Shown are the five homology domains: D1, β2a[1–17]); D2, β2a[18–178]; D3, β2a[179–213]; D4, β2a[214–415]; and D5, β2a[415–604], of which the D2 and D4 domains are defined by their high, *ca.* 75% sequence conservation among the type 1, 2, 3, and 4 calcium channel β subunits. Numbers correspond to amino acid positions at domain interfaces. (*E*) Outline of experiment. (*F*) SDS/PAGE and autoradiograms of ^{35}S-labeled β2a fragments synthesized by reticulocyte lysates (*Left* and *Center*) and binding to α_{1E}[CC14] fused to GST.

homologous to the Gβγ-binding domain of α_{1E}. Fragments containing these α_{1B} and α_{1A} sequences, expressed as GST-fusion proteins, were able to bind Gβγ (Fig. 1*E*). This indicated that not only the α_{1E} channels but also the N-type α_{1B} and P/Q-type α_{1A} channels have two Gβγ-binding sites. We propose that, as is the case for α_{1E}, Gβγ inhibits α_{1B} and α_{1A} also through its interaction with these C-terminal binding sites instead of the L1 sites. None of the C-terminal Gβγ-binding fragments of α_1 subunits contains a QXXER motif of the type found in the Gβγ-binding domains of type-2 adenylyl cyclase (AC2), the G protein-sensitive, inwardly rectifying potassium channel (GIRK1) and the C terminus of the Gβγ-responsive β adrenergic receptor kinase (βARK) (48). Further studies are needed to better define structural features of Gβγ-binding domains.

The location of the functionally relevant Gβγ-binding site in α_{1E} is of interest, because, as mentioned above and reported recently (12), α_{1E} has two independently identifiable binding sites for calcium channel β subunits: one is located in its L1 region as shown previously for α_{1A}, α_{1B}, and α_{1C} (28), and the other is in the α_{1E}[CC] fragment that also contains the Gβγ-binding domain. Using the strategy outlined in Fig. 4*A* and *B*, we then tested which subregion of the α_{1E}[CC] fragment binds β2a and found it to be the same as the one that binds Gβγ (i.e., α_{1E}[CC14]; Fig. 4*C*). Amino acid alignments of the four CCβ subunits defines five homology domains of which domains 1, 3, and 5 vary substantially in sequence, whereas domains 2 and 4 are highly conserved. Fig. 4 *D–F* shows that the portion of β2a that binds to α_{1E}[CC14] is β2a[206–415]. This corresponds to its homology domain 4 (D4) and is the same region of CCβ subunits that interacts with the L1 segments of α_1 subunits (49). Given the functional antagonism between Gβγ and CCβ (refs. 22, 24, and 25; see also Fig. 2*A.2*), we tested whether binding of one interferes with that of the other. For this purpose we synthesized in *E. coli* and purified several fragments of β2a. Two that contained domain 4 of β2a (β2a[D4] and β2a[D4–5]) bound to α_{1E}[CC14]; one that did not contain this domain, i.e., β2a[D1–3], did not bind to the C-terminal Gβγ-binding domain of α_{1E} (Fig. 4 *D–F*). β2a[D4] was then used to test for its ability to interfere with the binding of Gβγ to α_{1E}[CC14] fused to GST (Fig. 5). Binding of Gβγ was monitored by Western blotting after elution from the immobilized α_{1E}[CC14]. As shown in Fig. 5*C*, β2a[D4] prevented binding of Gβγ α_{1E}[CC14] in a concentration-dependent manner, whereas β2a[D1–3] did not.

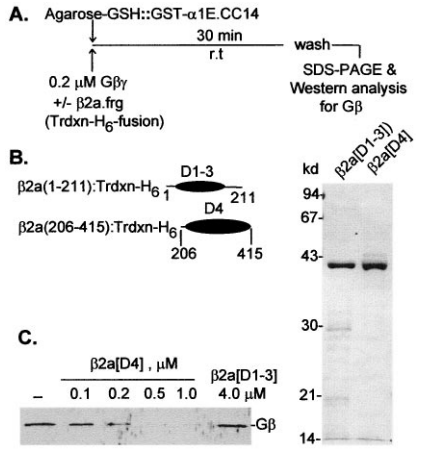

FIG. 5. Occlusion of the Gβγ-binding site by β2a[D4], the α_1-binding domain of, but not by, D1-D3 of β2a. (*A*) Outline of experiment. (*B*) Scheme of the structure of β2a[D1-D3] and β2a[D4] fusion proteins used and SDS/PAGE analysis of the purified fusion proteins. Fusion proteins were visualized by Coomassie blue staining. (*C*) Inhibition of Gβγ binding to α_{1E}[CC14] by increasing concentrations of the recombinant β2a[D4], but not by the recombinant β2a[D1-D3]. Gβ was visualized by Western blot analysis as in Fig. 1.

DISCUSSION

Taken together our experiments show that the molecular determinant that confers to α_{1E} the sensitivity to regulation by a G protein-coupled pathway resides in a short stretch of only 38 amino acids (α_{1E}[CC14]). A sequence homologous to α_{1E}[CC14] is present also in α_{1B} and α_{1A} Ca^{2+} channels, and both bind Gβγ (Fig. 1*D*).

Our data show further that Gβγ reduces macroscopic currents of α_{1E} by interacting with a site that is also seen by a stimulatory CCβ subunit. One mechanism by which Gβγ could be acting could have been by merely displacing a stimulatory β from its site. In this case, inhibition would have been the expression of a loss of β function. Given that the Gβγ-insensitive α_{1E}[DC277] retains all known regulations by β2a, this is not a likely mechanism. A different mechanism by which Gβγ might be acting is by enhancing an intrinsic inhibitory activity of the C terminus. In support of this possibility, in the case of α_{1C} channels, removal of 2/3 of its C terminus leads to an increase in channel activity due to an increase in the channel's Po, which is suggestive of existence of an intrinsic C terminus-mediated autoinhibitory activity (50). Studies of single-channel kinetics will be necessary to elucidate the biophysical nature of the changes induced in α_{1E} channels by Gβγ.

In summary we provide proof for direct interaction of Gβγ with two sites of the α_1 subunit of a neuronal, non-L-type Ca^{2+} channel and for the functional relevance of one but not the other of these sites. In addition, as shown in Fig. 5 and summarized in the model of Fig. 6, we showed the existence of direct antagonism between the binding of inhibitory Gβγ and that of a stimulatory CCβ subunit. Our results and conclusions stand in contrast to those of Zamponi *et al.* (26) and De Waard *et al.* (27), who have proposed the L1 Gβγ-binding site as the site responsible for mediation of inhibition by Gβγ. However, an analysis of their data shows that their conclusions were not based on unique interpretations of their data. Thus, Zamponi *et al.* (26) established only that L1 sequences that bind Gβγ can inhibit G protein regulation of α_{1B}. This result could also have been obtained with other Gβγ-scavenging compounds whether or not they were derived from α_1. De Waard *et al.* (27),

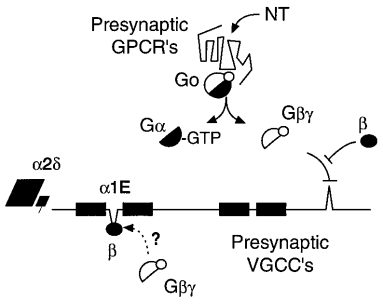

FIG. 6. Model of voltage-gated Ca^{2+} channels (VGCCs) regulated by protein–protein interactions defined in this report. The channels are envisioned as $\alpha_1.\beta.\alpha_2\delta$ heterotetramers regulated negatively by free Gβγ formed upon activation of a G protein of the G_i/G_o family. This occurs in response to stimulation of presynaptic G protein-coupled receptors (GPCRs) by the released neurotransmitter (NT) or by neuromodulatory peptides that are either coreleased with the neurotransmitter or released by neighboring neurons. The action of free Gβγ can in turn be prevented by a Ca^{2+}channel β subunit (CCβ). Note that the function of the Gβγ-binding site in L1 is unknown, and also that, although CCβ inhibition of the inhibitory effect of Gβγ is likely to be due to competitive displacement of Gβγ from its C-terminal binding site, we have not ruled out the possibility that CCβ interferes additionally by binding to Gβγ.

on the other hand, probed for a role of the L1-binding site by testing the effect of a point mutation that abolished $G\beta\gamma$ binding *in vitro*. But they did so using oocytes that were inhibited by G protein activation by only 12.6%, making it difficult to assess an effect of the mutation on inhibition of peak currents. Although De Waard *et al.* (27) attempted to circumvent this shortcoming in the assay system by measuring changes in kinetics (time to peak) and indeed seem to have observed the expected loss of an effect of G protein activation, studies by others (29, 30) have shown that the same mutation (QQIER to QQIEE) does not interfere with the inhibitory effect of $G\beta\gamma$. Our conclusion that the L1 site is not required for inhibition of the channel by $G\beta\gamma$ is based on the assessment of the unaltered regulation of EC30 by G protein activation, which is present even though this chimera carries an L1 loop that does not bind $G\beta\gamma$. It is worth noting that Zhang *et al.* (29) tested an α_{1B}/α_{1C} chimera equivalent to our EC30 and also found that it retained regulation by G protein activation.

The complete description of the biochemical pathway by which G protein-coupled receptors inhibit neurotransmission is now possible: neurotransmitter binds to the receptor, the receptor catalyzes the activation of a G protein, this results in GTP binding followed by dissociation of the trimeric protein into an α-GTP complex and a $\beta\gamma$ dimer, and, as surmised from intact cell studies by Ikeda (10) and Herlitze *et al.* (11), the $G\beta\gamma$ dimer proceeds to inhibit Ca^{2+} channel activation by the incoming action potential through direct interaction with its α_1 subunit. This inhibition can account for the potentiation of stimulus-evoked noradrenaline release from sympathetic terminals reported by Langer and Vogt (1) 25 years ago when they treated the synapses with phenoxybenzamine, an alkylating agent that irreversibly blocks α-adrenergic receptors. Likewise, this mechanism also explains the pertussis toxin-sensitive and, thus, G_i/G_o-mediated inhibition by both carbachol and morphine of the depolarization-evoked release of acetylcholine from rat myenteric plexus neurons (51).

Depending on the type of β subunit that colocalizes with α_1, and also on the type of α_1 subunit, it is possible to envision fine tuning of the inhibition by $G\beta\gamma$ to the extent that it may be extremely potent, as shown for inhibition of K^+-induced neurotransmitter release from cerebral cortex slices by opioids (3), or be very subtle and even absent. The G protein activated by receptor at the presynaptic terminal is likely to be G_o (reviewed in ref. 16).

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