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Mechanism of Action of G_q to Inhibit $G\beta\gamma$ Modulation of $Ca_V2.2$ Calcium Channels: Probed by the Use of Receptor-$G\alpha$ Tandems

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ABSTRACT

The stable interaction of a G-protein coupled receptor and a particular partner G-protein was made possible by creating tandems between the α_{2A} adrenergic receptor (α_{2A}-R) and pertussis toxin-resistant mutants of different $G\alpha$ subunits of heterotrimeric G-proteins. Both α_{2A}-R-$G\alpha_o$ and α_{2A}-R-$G\alpha_i$ proved able to reconstitute agonist-induced voltage-dependent inhibition of N-type calcium channels ($Ca_V2.2$) similar to the wild-type α_{2A}-R when expressed in COS-7 cells. The interaction of G_q with the $G_{i/o}$ signaling pathways was studied by expressing either $G\alpha_q$ or a chimeric construct based on $G\alpha_q$ containing the last five amino acids of $G\alpha_z$, which is activated by α_{2A}-R. It was found that $G\alpha_{qz5}$ activated by the wild-type α_{2A}-R inhibited $Ca_V2.2$ currents in a voltage-independent fashion. Furthermore, $G\alpha_{qz5}$ counteracted the voltage-dependent inhibition resulting from α_{2A}-R-$G\alpha_o$ activation. We subsequently investigated the basis for the behavior of $G\alpha_{qz5}$. Our evidence suggests that this occurs as a result of a downstream effect of activation of $G\alpha_{qz5}$ because it was blocked by C-terminal construct of phospholipase Cβ1. Furthermore it is likely to occur in part via protein kinase C (PKC) activation, because the PKC activator phorbol dibutyrate mimicked the effects of $G\alpha_{qz5}$ in α_{2A}-R-$G\alpha_o$-transfected cells. Conversely, cells expressing both α_{2A}-R-$G\alpha_o$ and $G\alpha_{qz5}$ exhibited a partial restoration of voltage-dependent inhibition in the presence of the PKC inhibitor bisindolylmaleimide I (GF 109203X). The potential sites of phosphorylation are discussed.

Calcium influx in any cell requires fine tuning to guarantee the correct balance between activation of calcium-dependent processes, such as muscle contraction and neurotransmitter release, and calcium-induced cell damage. G-protein-coupled receptors (GPCRs) play a role in negative feedback of the activity of voltage-dependent calcium channels (Dolphin, 1995). Establishing the basis for the specificity of the relationships between membrane receptors, G-proteins, and effectors has proven elusive, in part because of the promiscuity of the partners involved when expressed in heterologous systems. When different G-protein subunits are over-expressed together with GPCRs and calcium channels, the degree of specificity is rather low. For example, the α_{2A}-adrenergic receptor (α_{2A}-R) couples to all members of the $G_{i/o}$ family, including the pertussis toxin (PTX)-sensitive G_o and G_i, and the PTX-insensitive G_z (for review, see Hille, 1994).

In native systems, however, receptors display a more selective activation of endogenous G-proteins subtypes, with G_o being more important than G_i in the inhibition of calcium currents in sensory neurons (Campbell et al., 1993). Furthermore, in sympathetic neurons, muscarinic activation of G-protein-activated inward-rectifier (GIRK) channels is mediated by G_i, whereas muscarinic inhibition of N-type calcium channels is mediated by G_{oA} (Fernández-Fernández et al., 2001). These results point to the importance of the cellular localization of each receptor and G-protein subtype.

For GPCRs that associate with PTX-sensitive G-proteins, production of $G\beta\gamma$ dimers seems to be responsible for the direct voltage-dependent inhibition of N- and P/Q-type channels (Herlitze et al., 1996; Stephens et al., 1998), although it has also been proposed that in chick sensory neurons, $G\beta\gamma$ results in activation of PKC, to mediate the voltage-dependent inhibition caused by norepinephrine (Diversé-Pierluissi et al., 1995). Furthermore, $G\alpha$ subunits have also been im-

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ABBREVIATIONS: GPCR, G-protein-coupled receptor; α_{2A}-R, α_{2A}-adrenergic receptor; PTX, pertussis toxin; GIRK, G-protein-coupled inward rectifier K channel; GFP, green fluorescent protein; w.t., wild type; RS-79948-197, [8a*R*,12a*S*,13a*S*]5,6,8a,9,10,11,12,12a,13,13a-decahydro-12-ethanesulfonyl-3-methoxy-6H-isoquino[2,1-*g*]-[1,6]naphthyridine hydrochloride); GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; TTBS, Tween 20/Tris buffered saline; PDBu, phorbol dibutyrate; GF 109203X, bisindolylmaleimide I; PKC, protein kinase C; PLC-β1ct, phospholipase C-β1 C terminus; PP, prepulse; $G\alpha_t$, $G\alpha$-transducin; PIP_2, phosphatidylinositol 4,5-bisphosphate; IE, $Ile^{19}Ala$, $Glu^{20}Ala$.

plicated in mediating G-protein modulation (Diversé-Pierluissi et al., 1995).

One way to identify the direct effects of a specific G-protein on calcium channel activity is to link the G-protein α subunit to the receptor of choice to form a tandem construct. One of the advantages of this approach is the elimination of one of the signal amplification steps, occurring at the receptor/G-protein interaction level, because the two components are constrained to work with a 1:1 stoichiometry. Furthermore, there is increasing evidence against the established model which sees G-proteins shuttling between receptor and effector, and toward a view that there is a close localization of signal transduction elements in distinct membrane domains (Seifert et al., 1999). We used fusion proteins between the α_{2A}-R and either $G\alpha_{i1}$ or $G\alpha_{o1}$, both of which were rendered PTX-insensitive by means of a point mutation at residue 351 (Bahia et al., 1998). The Ile^{351} $G\alpha$ mutants were chosen over other possible PTX-resistant mutants because they resulted in the strongest activation by α_{2A}-R (Bahia et al., 1998). Activation of these tandems by the α_{2A}-R agonist clonidine was studied in COS-7 cells coexpressing N-type channels ($Ca_V2.2$) and comparing the response to that produced by the activation of the wild-type α_{2A}-R. These tandems have been found able to interact with endogenous G-proteins to a certain extent (Burt et al., 1998). In the present study, treatment of cells with PTX before recording allowed the receptor/G-protein tandems to be studied in isolation, effectively removing the contribution of endogenous $G_{i/o}$ proteins.

The carboxyl terminus of the $G\alpha$ subunit is not only a determinant of its sensitivity to PTX-dependent ADP-ribosylation but is also essential to confer specificity of coupling to GPCRs (Conklin et al., 1993). To examine whether $G\beta\gamma$ dimers liberated from G_q could also inhibit N-type Ca^{2+} channels, we exploited a chimeric $G\alpha_q$-protein. This construct was formed by a $G\alpha_q$ subunit in which the last 5 amino acids were substituted for the corresponding amino acids from $G\alpha_z$. The resulting $G\alpha_{qz5}$, unlike G_q itself, is able both to couple to the α_{2A}-R and to activate effectors specific to the G_q family, such as phospholipase C and the downstream protein kinase C (PKC) (Conklin et al., 1996). We report the effects of such a construct in isolation and when coexpressed with the α_{2A}-R-$G\alpha_o$ fusion protein and compare these effects with those of the wild type G_q subunit. The involvement of downstream effectors of $G\alpha_{qz5}$ is also examined.

Materials and Methods

Constructs. COS-7 cells were transiently transfected with the following cDNAs: rabbit $Ca_V2.2$ (GenBank accession no. D14157); rat β1b (GenBank accession no. X11394); and mut-3 green fluorescent protein (GFP).

The PTX-resistant α_{2A}-R-G-protein fusion proteins used throughout this study were prepared as described previously (Cavalli et al., 2000). In brief, Cys^{351} of rat $G\alpha_{i1}$ and $G\alpha_{o1}$ was mutated to Ile by site-directed mutagenesis and then used to create the α_{2A}-R-$G\alpha$ fusion proteins using porcine α_{2A}-R in pcDNA3. The Ile^{19}Ala, Glu^{20}Ala (IE) mutant of $G\alpha_{o1}$ was constructed, based on studies of an equivalent mutation (Ile^{25}Ala, Glu^{26}Ala) of $G\alpha_q$ (Evanko et al., 2000), and this was then incorporated into the PTX-resistant α_{2A}-R-$G\alpha_o$ fusion protein. The wild-type $G\alpha_q$ subunit ($G\alpha_q$ w.t.) and the $G\alpha_{qz5}$ subunits described previously (Conklin et al., 1993) were subcloned into pMT2. $G\alpha$-transducin ($G\alpha_t$) was in pcDNA3. The pEGFP-PLC-β1ct fusion construct of the C terminus of phospholipase $C\beta$ (PLC-β1ct) was described previously (Kammermeier and Ikeda, 1999).

Cell Culture and Transfections. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml) (all from Invitrogen, Paisley, UK) at 37°C, 5% CO_2, and passaged every 3 to 4 days. For transient transfections of the different constructs, a cDNA mixture was made containing the voltage-dependent calcium channel $Ca_V2.2$ subunit cDNA in a ratio of 3:1 with all the other constructs, β1b, α_{2A}-R, α_{2A}-R-G-protein tandems, and/or the $G\alpha$ subunits. Mut-3 GFP cDNA was also included at a ratio of 0.2. For transfection, 10 μl of GenePORTER reagent (Qbiogene, Harefield, UK) and 2 μl of cDNA mixture were preincubated in 1 ml of Dulbecco's modified Eagle's medium at 20°C for 1 h before addition to 35-mm Petri dishes containing approximately 2×10^6 cells. Cells were cultured at 37°C for 72 h, replated using a nonenzymatic cell dissociation medium (Sigma, Poole, UK), and maintained at 27°C for 1 to 8 h, before recording. PTX (Sigma) was used to inactivate the endogenous $G\alpha_{i/o}$ subunits by adding it to the culture medium at a concentration of 40 to 100 ng/ml for 16 h before replating the cells.

$[^3H]$RS-79948-197 Binding. To determine the levels of expression of the various α_{2A}-R-G-protein fusion proteins, the specific binding of $[^3H]$RS-79948-197 was measured as described previously (Ward and Milligan, 2002).

$[^{35}S]$GTPγS Binding. $[^{35}S]$GTPγS binding experiments were performed essentially as described for receptor-G-protein tandems incorporating $G\alpha_{11}$ (Carrillo et al., 2002). These were initiated by the addition of membranes containing 50 fmol of the fusion constructs to an assay buffer [20 mM HEPES, pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl, 1 μM guanosine 5′-diphosphate, 0.2 mM ascorbic acid, and 50 nCi of $[^{35}S]$GTPγS] in the absence or presence of clonidine (10 μM). Nonspecific binding was determined in the same conditions but in the presence of 100 μM GTPγS. Reactions were incubated for 15 min at 30°C and were terminated by the addition of 0.5 ml of ice-cold buffer containing 20 mM HEPES, pH 7.4, 3 mM $MgCl_2$, and 100 mM NaCl. The samples were centrifuged at 16,000*g* for 15 min at 4°C, and the resulting pellets were resuspended in solubilization buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA, and 1.25% Nonidet P-40) plus 0.2% SDS. Because all the α_{2A}-R-G-protein tandems used in these studies incorporated a hemagglutinin (HA) epitope tag at the N terminus of the receptor, samples were precleared with Pansorbin (Calbiochem, Nottingham, UK), followed by immunoprecipitation with the anti-HA antiserum 12CA5 (Roche Diagnostics, Lewes, UK). Finally, the immunocomplexes were washed twice with solubilization buffer, and bound $[^{35}S]$GTPγS was measured by liquid scintillation counting.

Immunoprecipitation and Immunodetection Studies. To analyze the interaction of α_{2A}-R-$G\alpha_o$ with $G\beta\gamma$ dimers, cells were transfected with α_{2A}-R-$G\alpha_o$ or α_{2A}-R-Ile^{19}Ala, Glu^{20}Ala $G\alpha_o$ in the absence or presence of plasmids encoding G-protein β1 and γ2 subunits. Cells were washed once with ice-cold phosphate-buffered saline and immediately homogenized in a lysis medium containing 50 mM HEPES, pH 7.4, 10 mM $Na_4P_2O_7$, 100 mM NaF, 10 mM EDTA, 0.1 mM Na_3VO_4, 1% Triton X-100, and a protease inhibitor cocktail (Complete; Roche). Cell lysates were centrifuged (15 min, 13,000 rpm) and the supernatants precleared for 1 h with nonspecific serum and protein A. Next, samples were incubated overnight with a polyclonal antiserum directed against the C-terminal decapeptide of $G\alpha_{o1}$ (Mullaney and Milligan, 1990). The immunocomplexes were then captured with protein A-agarose.

For immmunoblotting, cell lysates or immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes and blocked for 2 h with 5% nonfat dried milk in 0.05% Tween 20/Tris-buffered saline (TTBS). Then, the PVDF membranes were probed overnight at 4°C with an antiserum (BN) directed against the N-terminal decapeptide of the G-protein β1 subunit (Green et al., 1990) and washed with TTBS. The PVDF membranes were incubated for 20 min with horseradish peroxidase conjugated to anti-rabbit IgG (1:20,000) (Amersham Biosciences). Finally, they were washed with TTBS and developed by enhanced chemiluminescence.

Electrophysiology. Fluorescent COS-7 cells expressing GFP were chosen for whole-cell, patch-clamp recording. Borosilicate glass electrodes were used with a resistance of 2 to 5 MΩ when filled with a solution containing 140 mM cesium aspartate, 5 mM EGTA, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 2 mM K_2ATP, and 20 mM HEPES, pH adjusted to 7.2 with CsOH, 310 mOsM with sucrose. Cells were perfused with an extracellular solution containing 160 mM tetraethylammonium-Br, 2 mM KCl, 1.0 $NaHCO_3$, 1.0 $MgCl_2$, 10 mM HEPES, 4 mM glucose, and 10 mM $BaCl_2$, pH 7.4, 320 mOsM with sucrose. Barium currents were recorded using an Axopatch-1D amplifier (Axon Instruments, Union City, CA). Data were filtered at 2 kHz, digitized at 5 to 10 kHz, and analyzed using pCLAMP 6 (Axon Instruments) and Origin 5.0 (Microcal, Northampton, MA). Cell capacitance compensation and series resistance compensation between 65 and 80% were applied electronically. Records are shown after leak subtraction (P/4 or P/8 protocol).

Facilitation was assessed by using a double-pulse protocol (see Fig. 1a, top). A first 30-ms step (P1) usually to 0 mV was followed by a 300-ms period of repolarization to −100 mV. A strongly depolarizing prepulse PP of 30 to +100 mV was then delivered before a second pulse (P2) to the same voltage as the first test pulse, to assess the voltage-dependence of current inhibition. The PP and the second pulse were separated by a 10-ms repolarization time to −100 mV. Pulses were delivered every 15 s. Currents were measured 10 ms after the onset of both P1 and P2 and the average over a 2-ms period was calculated and used for subsequent analysis. The 300-ms interval between P1 and PP was sufficient to minimize the voltage-dependent calcium channel inactivation caused by P1. The duration and amplitude of the PP were chosen to produce maximal facilitation in the conditions used (data not shown). Experiments were performed at room temperature (20–24°C). Drugs were applied by the use of a gravity-fed, electronically controlled, multibarrelled perfusion system. Current density-voltage (I-V) relationships were fitted with a modified Boltzmann equation as follows: $I = G_{max}(V - V_{rev})/(1 + \exp(-(V - V_{50,act})/k))$, where I is the current density (picoamperes per picofarad), G_{max} is the maximal conductance (nanosiemens per picofarad), V_{rev} is the reversal potential, $V_{50,act}$ is the mid-point voltage for current activation, and k is the slope factor.

The time constant of activation (τ_{act}) was calculated by fitting a single exponential to the current traces: $I = A \times \exp(-t/\tau_{act}) + C$, where A is the amplitude of the component with time constant τ, and C is a constant. Data are expressed as mean ± S.E.M., and statistical significance between conditions was examined using Student's t test or paired t test, as appropriate.

Materials. [^{3}H]RS-79948-197 (90 Ci/mmol) was from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK), [^{35}S]GTPγS (1250 Ci/mmol) was from PerkinElmer Biosciences (Warrington, UK). Clonidine hydrochloride (Calbiochem) was prepared as a 10^{-2}

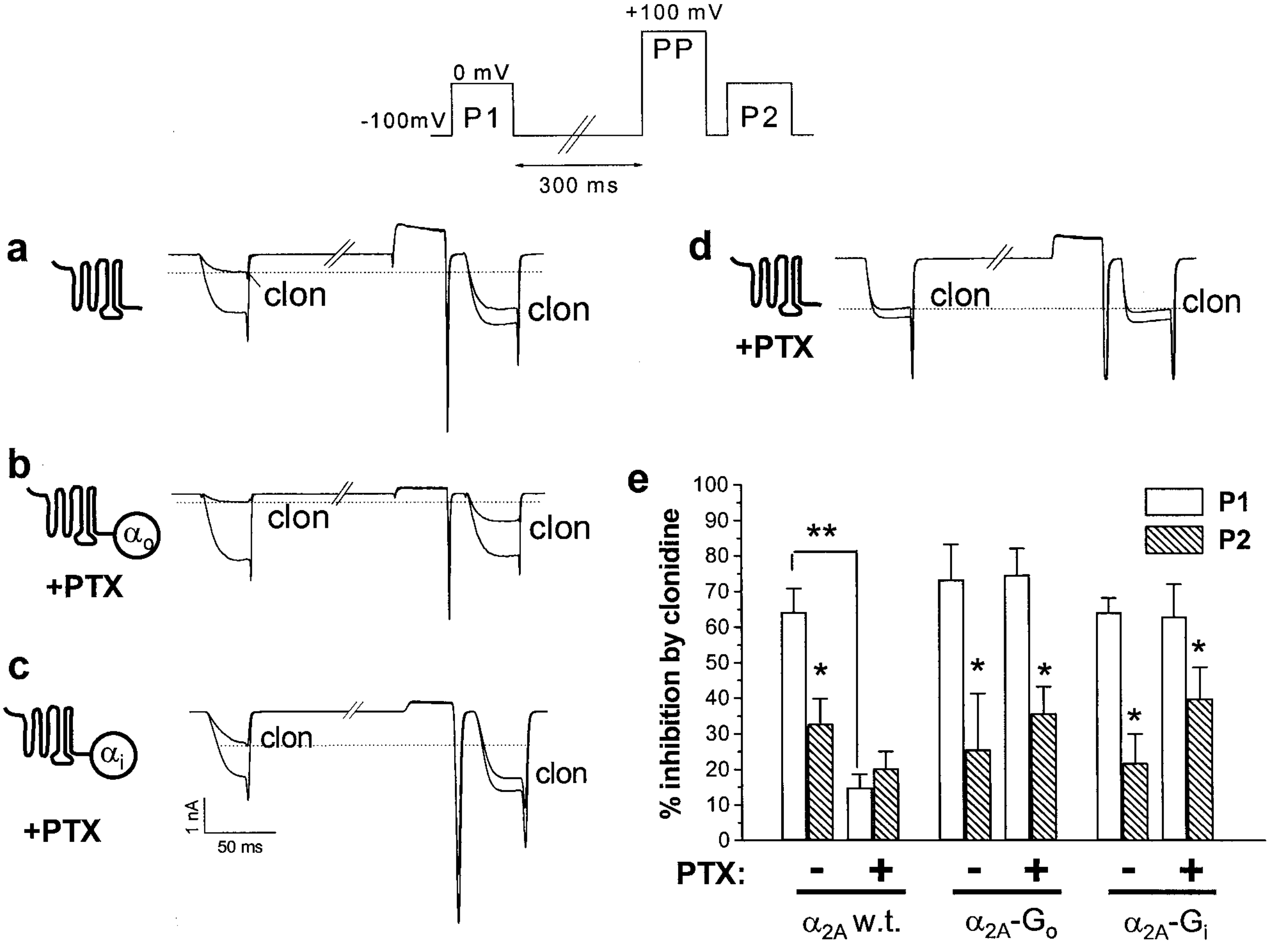

Fig. 1. Comparison of inhibition of I_{Ba} by α_{2A}-R and α_{2A}-R-$G_{i/o}$ tandems. Top, double-pulse voltage-clamp protocol used to measure the PP facilitation of I_{Ba}. Two 30-ms test pulses (P1 and P2) to 0 mV were separated by 300-ms repolarization to −100 mV, a 50-ms PP to +100 mV and a 10-ms period of repolarization to −100 mV. Recordings were made every 15 s. a–d, schematic representation of the α_{2A}-R constructs is given on the left. Example recording from cells expressing different receptor constructs. Currents recorded in control and after application of 10 μM clonidine are superimposed. a, the α_{2A}-R w.t.; b, the PTX-resistant α_{2A}-R-Gα_o; c, the PTX-resistant α_{2A}-R-Gα_i; d, example traces after preincubation with PTX from a cell expressing α_{2A}-R w.t.; e, summary of I_{Ba} inhibition by clonidine before (P1, □) and after (P2, ▨) the depolarizing PP. Values are reported without and with pretreatment with PTX for the α_{2A}-R w.t. (n = 4 and 9, respectively), and for the receptor tandems α_{2A}-R-Gα_o (n = 5 and 18, respectively) and α_{2A}-R-Gα_i (n = 8 for both) (*, $p < 0.05$; **, $p < 0.01$; either paired t test, between P1 and P2, or unpaired t test between ± PTX, as indicated).

M stock in H_2O. The protein kinase C activator phorbol-12,13-dibutyrate (PDBu; Calbiochem) and the PKC inhibitor bisindolylmaleimide I (GF 109203X, Calbiochem) were prepared as 10^{-2} M stock in DMSO. All drugs were diluted in the experimental solutions to the final concentrations indicated.

Results

Effect of the α_{2A} Adrenergic Receptor-Gα_i and -Gα_o Tandems. We first expressed either α_{2A}-R w.t. or the PTX-insensitive receptor-Gα tandems α_{2A}-R-Gα_{o1}C^{351}I (α_{2A}-R-Gα_o) or α_{2A}-R-Gα_iC^{351}I (α_{2A}-R-Gα_i) together with the $Ca_V2.2$ calcium channel. The inhibition of the expressed Ba^{2+} currents (I_{Ba}) by activation of the α_{2A}-R w.t. was compared with the effect of the receptor G-protein tandems (Fig. 1). Overall, the α_{2A}-R agonist clonidine (10 μM) inhibited N-type I_{Ba} via activation of both the free α_{2A}-R and the tandem α_{2A}-R-Gα constructs, as exemplified by the current traces in Fig. 1, a−c. The inhibition was rapid (< 15 s) and reversible upon washing (data not shown). The extent of I_{Ba} inhibition at 0 mV is given in Fig. 1e (□). In the absence of PTX, I_{Ba} was similarly reduced by both the wild-type α_{2A}-R (64.2 ± 6.6%, n = 9, Fig. 1a) and the tandems α_{2A}-R-Gα_o (77.6 ± 6.6%, n = 5, Fig. 1b) and α_{2A}-R-Gα_i (64.1 ± 4.0%, n = 8, Fig. 1c). Thus, removal of the amplification step between receptor and G-protein did not affect the ability of $G_{i/o}$ to produce inhibition of $Ca_V2.2$ I_{Ba}.

It has been observed previously that chimeric receptor-Gα constructs are able to activate not only the tethered Gα subunit but also endogenous subunits of the $G_{i/o}$ family (Burt et al., 1998). The use of PTX therefore allows isolation of the effects of exogenous Gα subunits mutated to be PTX-resistant by rendering the endogenous $G_{i/o}$ subunits unable to couple to the receptor. Preincubation of the cells with PTX greatly reduced the inhibition produced by the α_{2A}-R w.t. (see traces in Fig. 1d and mean results in Fig. 1e). Conversely, PTX did not significantly affect the functioning of the two PTX-insensitive receptor G-protein tandems. The calcium channel currents at 0 mV were still reduced by 74.1 ± 6.5% (n = 18, Fig. 1b) and 62.9 ± 9.1% (n = 8, Fig. 1c) with the Gα_o and the Gα_i fusion proteins, respectively, after pretreatment with the toxin (Fig. 1e). Experiments repeated with a lower concentration of clonidine (100 nM) gave comparable results in terms of degree of inhibition, demonstrating that maximal receptor activation was achieved at the concentration of agonist used (data not shown).

Inhibition of N-type currents by the receptor-G$\alpha_{i/o}$ tandems was largely voltage-dependent, as seen by using a double pulse voltage-clamp protocol (Fig. 1, a—-d). The PP was able to reverse the agonist-induced inhibition induced by either α_{2A}-R w.t. (Fig. 1a) or the α_{2A}-R-Gα_o (Fig. 1b) and α_{2A}-R-Gα_i (Fig. 1c) tandems, whereas incubation with PTX eliminated the voltage-dependent effects of the α_{2A}-R w.t. (Fig. 1d). The amount of inhibition by clonidine before and after the PP is summarized in Fig. 1e. The resultant "facilitation" (determined as the P2 current amplitude divided by P1 current amplitude) was substantial for all three receptor constructs. In all cases, however, removal of inhibition during P2 by the PP to +100 mV was never complete, indicating a voltage-independent inhibitory component.

As a corollary of the voltage-dependence of the inhibition of I_{Ba} by clonidine, it should also be abolished at large step potentials. The voltage-clamp protocol used to examine this was similar to that shown in Fig. 1 with the exception that both test pulses (P1 and P2) were varied from −40 to +70 mV in 10-mV increments. Example traces are shown in Fig. 2a, whereas the mean I-V plots for values measured in P1, before and during application of clonidine, for cells expressing the α_{2A}-R-Gα_o (n = 8) are shown in Fig. 2b. With all receptor constructs, the agonist caused both a reduction in I_{Ba} and a depolarizing shift in the I-V relationship. The $V_{50,act}$ during P1 was significantly depolarized for cells expressing α_{2A}-R-Gα_o, from −6.4 ± 3.1 to +9.0 ± 4.0 mV ($p < 0.05$, n = 6, Fig. 2b) and, for cells expressing α_{2A}-R-Gα_i, from +2.5 ± 2.4 to +9.7 ± 0.7 mV ($p < 0.05$, n = 6). No significant differences in the V_{rev} or in the G_{max} were detected (Fig. 2b; data not shown). The P2/P1 facilitation ratios for the different test potentials are reported in Fig. 2, c–d. The PP revealed some tonic facilitation in the absence of the agonist (□), which was more marked when expressing the α_{2A}-R w.t., where P2/P1 was 2.3 ± 0.3 at 0 mV (Fig. 2c). Clonidine enhanced the voltage-dependent facilitation, although the effects were much greater for the α_{2A}-R-Gα_o tandem than for the α_{2A}-R w.t. (Fig. 2d). Maximal facilitation was obtained at −10 or 0 mV and it was absent above +20 mV.

Not only did activation of the α_{2A}-R-Gα tandems cause a reduction in current amplitude but the activation phase of the current was typically slowed during P1; this effect was reversed by the PP (e.g., Fig. 1, a–c). For example, for those cells transfected with the α_{2A}-R-Gα_o tandem, the τ_{act} at 0 mV during P1 was 3.7 ± 0.5 ms in control and 6.1 ± 1.1 ms during clonidine application (n = 10, $p < 0.05$, see Fig. 1b). This slowed activation was reversed by Gα_t, which acts as a G$\beta\gamma$ sink to sequester free G$\beta\gamma$ subunits but does not couple to the α_{2A}-R. Example traces are shown in Fig. 3a (top). After cotransfection of Gα_t with α_{2A}-R-Gα_o, there was no longer a difference in the τ_{act} values measured in control and clonidine during P1 (2.9 ± 0.6 ms and 3.4 ± 0.5 ms, respectively, n = 9). Along with this effect, Gα_t was able significantly to reduce inhibition by clonidine at 0 mV from 74.1 ± 6.5 to 43.0 ± 6.7% ($p < 0.001$; Fig. 3b) and to reduce the P2/P1 facilitation ratio in the presence of clonidine to 1.54 ± 0.24 at 0 mV, although this was still significantly greater than the P2/P1 ratio under control conditions (Fig. 3c).

Given that these data were obtained in the presence of PTX, to prevent promiscuous coupling of the tandems to additional endogenous Gi/o proteins, these findings indicate that the α_{2A}-R tandems are able to reconstitute inhibitory effects on $Ca_V2.2$ calcium channel currents by means of the tethered G$\alpha_{i/o}$ that are almost identical to the wild-type receptor coupling to endogenous G-proteins and that such effects are very likely to be mediated purely by G$\beta\gamma$ dimers. It has been found previously that mutation of both Ile^{25} and Glu^{26} of $G_q\alpha$ to Ala severely limits interaction with the G$\beta\gamma$ complex (Evanko et al., 2000). These residues are highly conserved in other G-protein α subunits. We thus constructed a form of the PTX-resistant α_{2A}-R-Gα_o tandem (IE) that also incorporated the equivalent mutations of Ile^{19}Ala and Glu^{20}Ala in Gα_o. Application of clonidine to cells expressing the IE form of the α_{2A}-R-Gα_o tandem produced no inhibition of I_{Ba}, and no effect on facilitation (Fig. 3, a, bottom, and b–c). It is also evident that these $Ca_V2.2$ currents show some tonic modulation, being slowly activating and facilitated by a prepulse, although this is no greater than for the free α_{2A}-R (Fig. 2c).

To examine the binding of G$\beta\gamma$ to the IE mutant of α_{2A}-R-Gα_o, either α_{2A}-R-Gα_o or the IE form of this construct was cotransfected together with plasmids encoding the Gβ1 and Gγ2 subunits. Cell lysates were subsequently immunoprecipitated with an antiserum (OC) that identifies the C-terminal decapeptide of Gα_{o1}. Such samples were then resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with an antiserum (BN) that identifies the N-terminal decapeptide of Gβ1. Although the α_{2A}-R-Gα $_o$ tandem allowed coimmunoprecipitation of β1 subunit (Fig. 3d, lane 2), this was not observed for the IE form of the tandem receptor (Fig. 3d, lane 3).

Investigation of α_{2A}-R-Gα_q and α_{2A}-R-Gα_{qz5} Chimeras. Because the expression of the receptor/G-protein tandems indicated that the release of activated Gα subunits, Gα_i and Gα_o, does not play any direct role in G-protein-effector coupling for calcium channel inhibition, we were interested in studying whether G$\beta\gamma$ released from another class of G-protein, G$_q$, could also participate in the inhibitory process. However G$_q$ is known not to couple efficiently to the α_{2A}-R (Dorn et al., 1997). To use the same receptor for activation of both G$_{i/o}$ and G$_q$ pathways, we therefore employed the chimeric construct Gα_{qz5}. This subunit conserved the main structure of Gα_q but the last five amino acids were substituted for those of Gα_z, a PTX-resistant member of the G$_{i/o}$ family that does couple to the α_{2A}-R (Conklin et al., 1993). Tandem α_{2A}-R-Gα_q and α_{2A}-R-Gα_{qz5} constructs were assembled, and their functionality was assessed biochemically.

Evidence of the activation of the PTX-resistant G-proteins within the α_{2A}-R-Gα tandems by clonidine was obtained by monitoring agonist-induced binding of [^{35}S]GTPγS. Expression levels of the α_{2A}-R-containing fusion proteins in membranes of PTX-treated cells were quantified by saturation ligand binding studies employing the high-affinity α_2-adrenoceptor antagonist [^{3}H]RS-79948-197. [^{35}S]GTPγS binding studies were performed in the presence and absence of clonidine (10 μM) on membrane fractions expressing equal amounts of the various fusion proteins. After this, the anti-HA antibody 12CA5 was used to immunoprecipitate the samples, because all of these constructs contained an N-terminal HA epitope tag. Significant levels of [^{35}S]GTPγS binding were observed for both the Gα_o- and Gα_i-containing

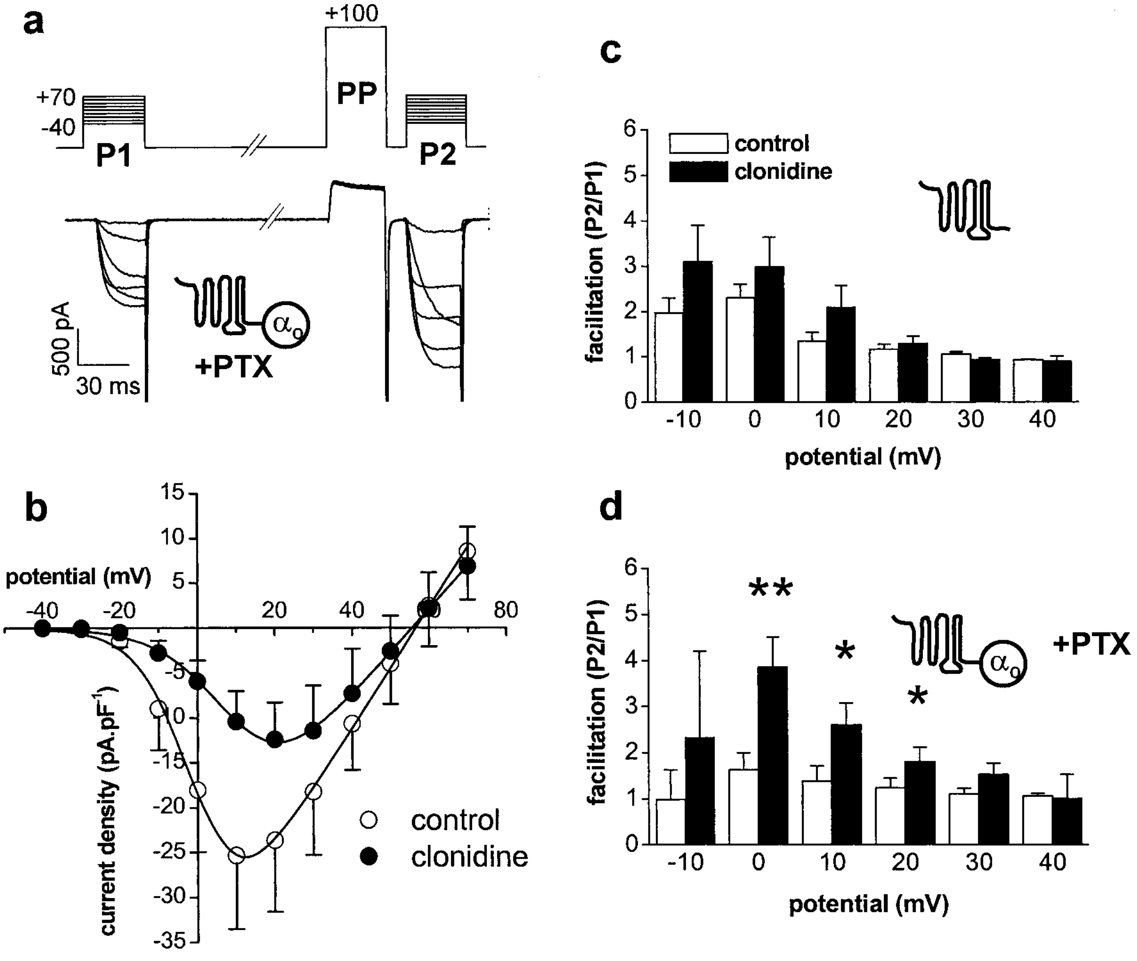

Fig. 2. Effect of varying the test potential on I_{Ba} inhibition by receptor-G$_{i/o}$ tandems. a, top, voltage-clamp protocol. Both P1 and P2 were varied from −40 to +70 mV in 10 mV increments. Bottom, an example of superimposed traces recorded in the presence of clonidine from a cell expressing α_{2A}-R-Gα_o and treated with PTX. b, I-V relationship for cells expressing α_{2A}-R-Gα_o before the PP. The data are average values of current density before (○) and after (●) application of clonidine ($n = 8$). I-V plots were fitted with a modified Boltzmann equation (see *Materials and Methods*). c and d, values of I_{Ba} facilitation ratios (P2/P1) in control (☐) and in the presence of clonidine (■), for the α_{2A}-R w.t. in the absence of PTX ($n = 4$) (c) and α_{2A}-R-Gα_o treated with PTX ($n = 8$) (d). Only the values for voltages between −10 and +40 mV are reported. Statistical significances of the effect of clonidine: *, $p < 0.05$; **, $p < 0.01$, paired *t* test.

fusion proteins; this was stimulated markedly by the presence of clonidine (Fig. 4). In contrast, little binding of $[^{35}S]GTP\gamma S$ was observed to the α_{2A}-R-Gα_q and α_{2A}-R-Gα_{qz5} constructs, even in the presence of clonidine, consistent with a lack of activation of these G-proteins by the associated α_{2A}-R. The inability of clonidine to promote binding of $[^{35}S]GTP\gamma S$ to the fusion proteins containing Gα_q does not reflect the well appreciated difficulty in monitoring nucleotide exchange for such G-proteins in standard $[^{35}S]GTP\gamma S$ binding assays. We have recently shown that combination of use of receptor-G-protein tandems and selective immunoprecipitation allows a 30-fold stimulation of binding in the presence of agonist when such G-proteins are linked in tandem with appropriate receptors (Carrillo et al., 2002). A preliminary investigation also failed to show any clonidine-mediated inhibition of $Ca_V2.2$ via the α_{2A}-R-Gα_{qz5} tandem, but because

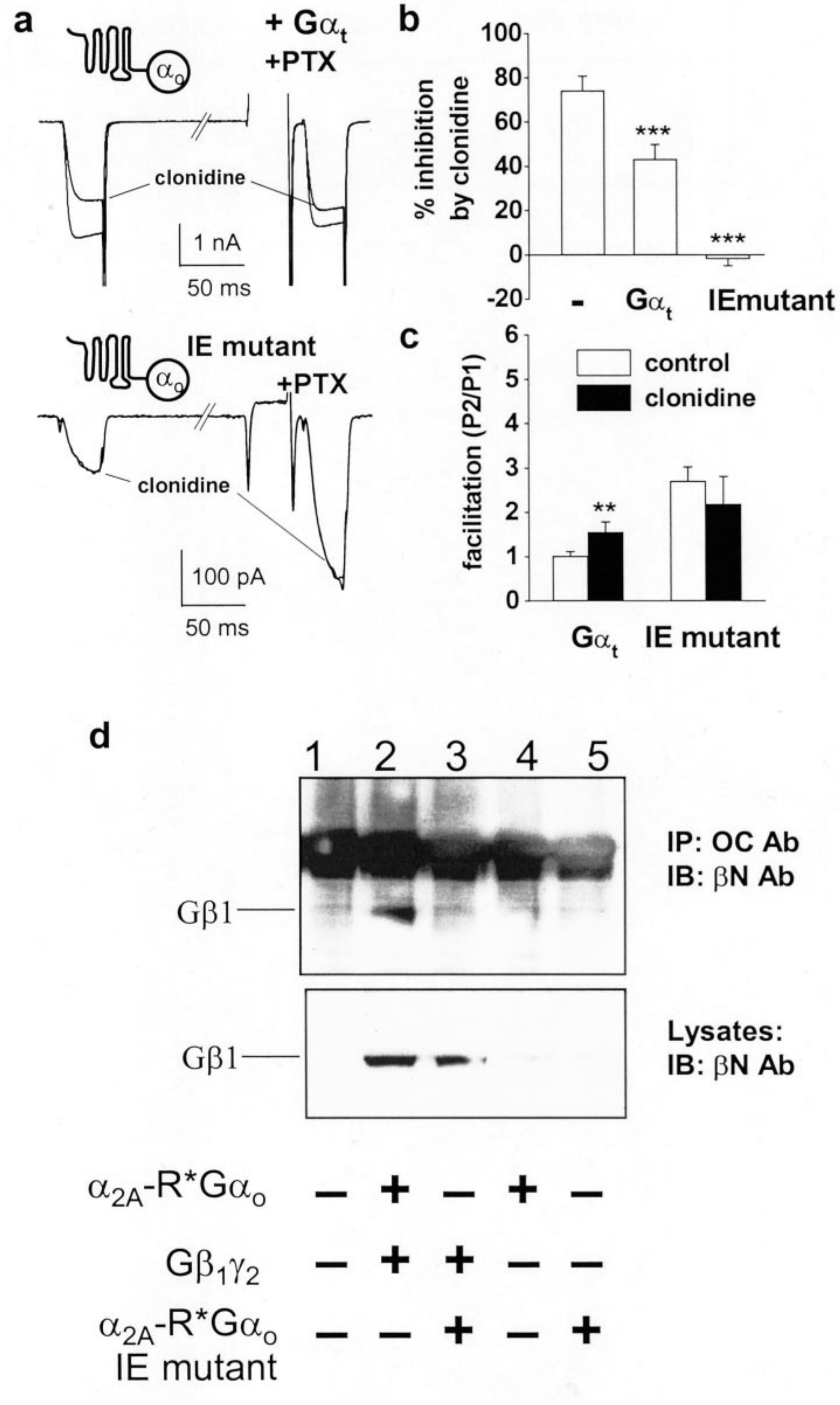

Fig. 3. The effects of α_{2A}-R-Gα_o are mediated by G$\beta\gamma$. A, example traces recorded in cells expressing the α_{2A}-R-Gα_o tandem and Gα_t (upper traces) or the IE mutant of α_{2A}-R-Gα_o (lower traces). b, inhibition by clonidine in cells expressing α_{2A}-R-Gα_o alone ($n = 10$), α_{2A}-R-Gα_o, and Gα_t ($n = 9$) or the IE mutant of α_{2A}-R-Gα_o ($n = 3$). Statistical significance, ***, $p < 0.001$ compared with control, Student's *t* test. c, facilitation ratios for the same cells as in b. Statistical significance, **, $p < 0.01$ compared with control, paired *t* test. d, mutation of Ile^{19} and Glu^{20} of Gα_o inhibits interaction with the G-protein $\beta 1$ subunit. Cells were mock transfected (lane 1) or transfected with either the α_{2A}-R-Gα_o fusion protein (lanes 2, 4) or the α_{2A}-R-(Ile^{19}Ala, Glu^{20}Ala)−Gα_o fusion (IE mutant, lanes 3 and 5). In lanes 2 and 3, cells were also transfected with plasmids encoding Gβ1and Gγ2. Top, samples were immunoprecipitated with antiserum OC against the C-terminal of Gα_{o1}, resolved by SDS-PAGE, and immunoblotted with an antiserum against the Gβ1 subunit. Bottom, lysates from the cells were resolved by SDS-PAGE and immunoblotted to detect expression of the β1subunit. Data are from a representative experiment.

these receptor-Gα_q tandems were nonfunctional biochemically, their coupling to Ca$_V$2.2 was not further examined.

We therefore employed free Gα_q and Gα_{qz5} to examine whether G$\beta\gamma$ released from G$_q$ or G$_{qz5}$ can signal to N-type calcium channels (Fig. 5a). We confirmed, by coexpressing the α_{2A}-R w.t. with Gα_q w.t. in cells treated with PTX, that Gα_q did not couple directly to the α_{2A}-R. Perfusion of clonidine induced only 7.1 ± 1.1% reduction in the current (n = 5, Fig. 5b). In contrast, expression of Gα_{qz5} with the α_{2A}-R w.t. resulted in significantly greater inhibition of Ca$_V$2.2 currents by clonidine (35.8 ± 8.6%, n = 9, Fig. 5, a and b). Surprisingly however, this was not removed by a PP to +100 mV, the inhibition in P2 being 34.5 ± 5.4% (n = 9, Fig. 5b). Thus, the inhibition elicited by Gα_{qz5} was much greater than that elicited by Gα_q w.t. ($p < 0.001$) but was not voltage-dependent. The P2/P1 facilitation ratio in the presence of Gα_{qz5} was around unity and was unaffected by the presence of agonist (0.98 ± 0.07 in control, 1.20 ± 0.23 in clonidine, $p > 0.05$, Fig. 5c). Current traces in the presence of Gα_{qz5} showed no evidence of slowing of the kinetics of activation in response to clonidine (e.g., traces in Fig. 5a and data not shown). To determine whether voltage-dependent inhibition was completely absent for Gα_{qz5}, we also examined the voltage-dependence of inhibition over a range of potentials. However, no obvious facilitation was evident at any test potential (data not shown). These results demonstrate that the C-terminal modification of G$_q$ allowed Gα_{qz5} to couple to the α_{2A}-R, causing a reduction in I$_{Ba}$, although the inhibition was voltage-independent and smaller than that elicited by the tandems α_{2A}-R-Gα_o and α_{2A}-R-Gα_i or the wild type α_{2A}-R coupling to endogenous G-proteins.

Interaction between α_{2A}-R-Gα_o and Gα_{qz5}. It has been observed previously that chimeric receptor-Gα constructs are able to activate not only the tethered Gα subunit but also endogenous subunits of the G$_{i/o}$ family (Burt et al., 1998). Accordingly, Gα_{qz5} might be expected also to interact with, and to be activated by, the α_{2A}-R-Gα_o tandem used in this part of the study. We investigated this potential interaction by coexpressing the tandem α_{2A}-R-Gα_o with the Gα_{qz5} subunit, and treating all cells with PTX.

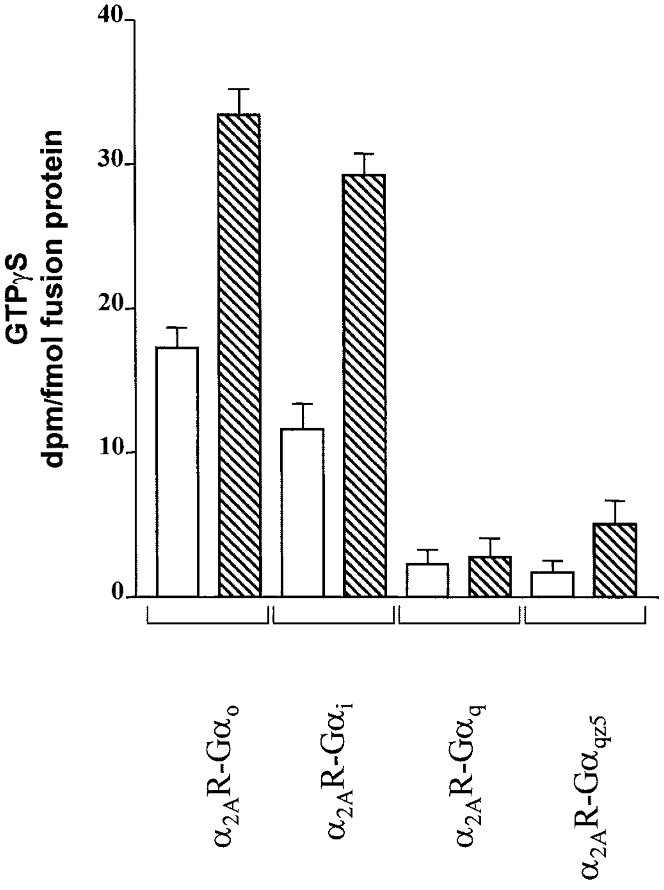

Fig. 4. Clonidine stimulates binding of [^{35}S]GTPγS to fusion proteins between the α_{2A}-R and both Gα_{o1} and Gα_{i1}. Membranes were prepared from cells transfected to express fusion proteins between an N-terminally HA-tagged form of the α_{2A}-R and each of (Cys^{351}Ile) Gα_o, (Cys^{351}Ile) Gα_i, Gα_q, or Gα_{qz5}. After [^{35}S]GTPγS binding assays performed in the absence (☐) or presence (▨) of clonidine (10 μM), samples were immunoprecipitated with the anti-HA antibody 12CA5 and ^{35}S content was determined. Data represent mean ± S.E.M. (n = 3).

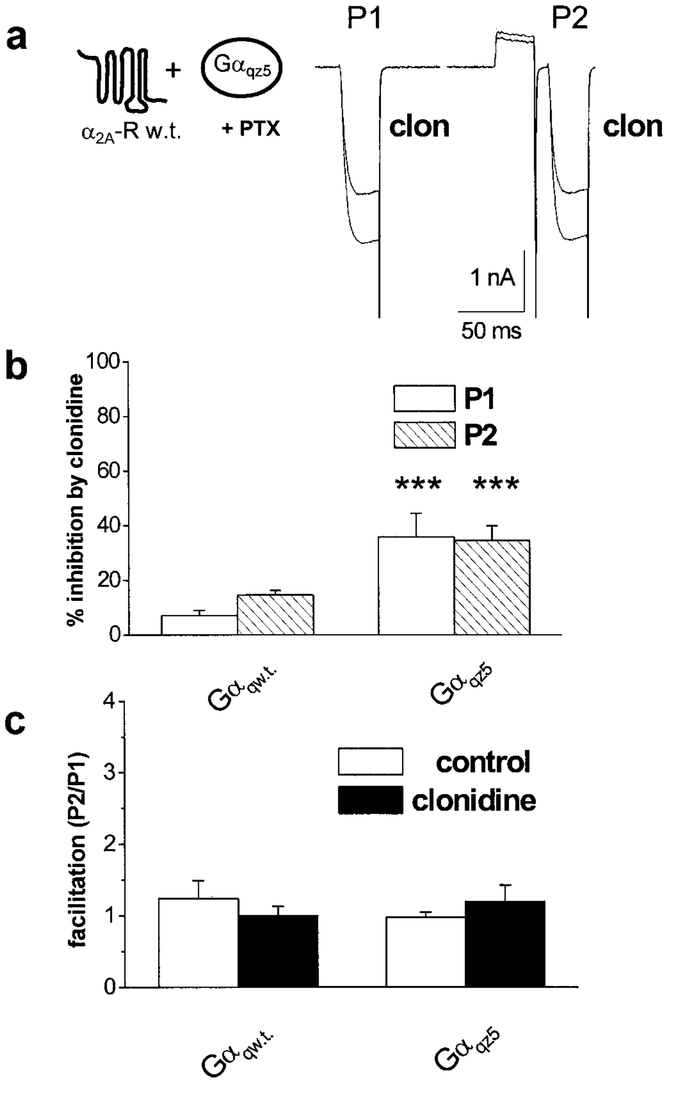

Fig. 5. The ability of the Gα_{qz5} subunit to support G-protein modulation of Ca$_V$2.2 currents. a, example traces recorded in the presence and absence of clonidine from a cell expressing the α_{2A}-R w.t. and Gα_{qz5}. b, inhibition by clonidine in cells expressing the α_{2A}-R w.t. with the Gα_q w.t. subunit (n = 5, left) or the α_{2A}-R w.t. with the Gα_{qz5} subunit (n = 9, right). ☐, inhibition during P1; ▨, inhibition during P2. c, facilitation (P2/P1 ratio) of currents in control (☐) and after application of clonidine (■) for the same combinations of constructs as in b. All cells were pretreated with PTX. Statistical significance compared with Gα_q w.t., ***, $p < 0.001$, Student's t test.

The first observation was that the inhibition of I_{Ba} obtained when coexpressing α_{2A}-R-$G\alpha_o$ with $G\alpha_{qz5}$ was significantly smaller than in cells expressing α_{2A}-R-$G\alpha_o$ alone (Fig. 6a). Inhibition was 21.0 ± 12.4% in P1 ($n = 16, p < 0.01$, Fig. 6b). Interestingly, the presence of $G\alpha_{qz5}$ also almost abolished facilitation by the PP at all potentials examined (Fig. 6c). For example the P2/P1 facilitation ratio in clonidine was 1.24 ± 0.38 at 0 mV and 0.87 ± 0.11 at +10 mV ($n = 11$, both $p < 0.01$ compared with the much greater facilitation shown by the α_{2A}-R-$G\alpha_o$ alone). As a corollary of this, no agonist-

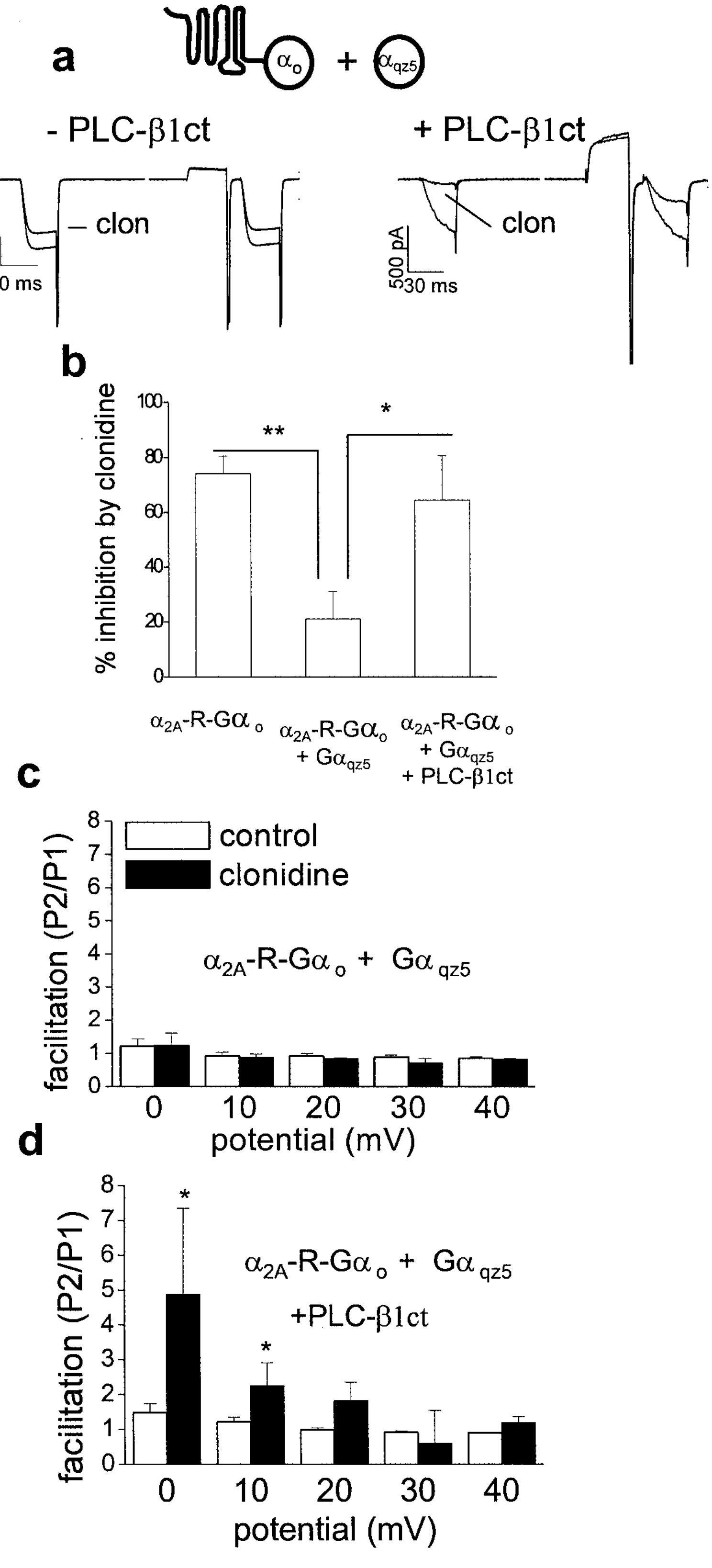

Fig. 6. The effect of $G\alpha_{qz5}$ is counteracted by a C terminal construct of phospholipase C-β1. a, example recordings from cells expressing both the tandem α_{2A}-R-$G\alpha_o$ and the $G\alpha_{qz5}$ subunit without (left) and with (right) the additional presence of PLC-β1ct. The voltage protocol is that shown in Fig. 1. b, mean percentage inhibition by clonidine for α_{2A}-R-$G\alpha_o$ alone ($n = 18$); α_{2A}-R-$G\alpha_o$ and $G\alpha_{qz5}$ ($n = 16$); and α_{2A}-R-$G\alpha_o$, $G\alpha_{qz5}$, and PLC-β1ct ($n = 6$). Statistical significance, **, $p < 0.01$; *, $p < 0.05$ as indicated. c, voltage-dependence of facilitation ratio for coexpression of α_{2A}-R-$G\alpha_o$ and $G\alpha_{qz5}$ ($n = 11$). Voltage protocol as in Fig. 2a. d, voltage-dependence of facilitation ratio for coexpression of α_{2A}-R-$G\alpha_o$, $G\alpha_{qz5}$, and PLC-β1ct ($n = 6$). Voltage protocol as in Fig. 2a. Statistical significance, *, $p < 0.05$, compared with the P2/P1 ratio in clonidine for α_{2A}-R-$G\alpha_o$, $G\alpha_{qz5}$ in the absence of PLC-β1ct (given in Fig. 6c).

induced depolarizing shift of the I-V relationship for I_{Ba} was detected (data not shown). Furthermore, no slowing of the activation kinetics was evident during P1 (e.g., Fig. 6a). In summary, coexpression of $G\alpha_{qz5}$ with α_{2A}-R-$G\alpha_o$ reduced the inhibition and reversed the P2/P1 facilitation observed upon activation of the α_{2A}-R-$G\alpha_o$ alone.

Mechanism of Action of $G\alpha_{qz5}$. We addressed the possibility that the effects produced by $G\alpha_{qz5}$ on $Ca_V2.2$ channel modulation might be mediated by a signaling pathway downstream from G_q rather than directly by the $G\alpha_{qz5}$ subunit. It has been proposed that overexpression of any $G\alpha$ subunit could abolish calcium channel inhibition by sequestering $G\beta\gamma$ subunits, which would therefore become unavailable for receptor activation (Jeong and Ikeda, 1999). However, this will depend on the balance between $G\alpha$ activation to form free $G\alpha$-GTP and $G\beta\gamma$ interaction with the $G\alpha$-GDP species. In such a scenario, coexpression of $G\alpha_{qz5}$ could buffer the effect of the $G\beta\gamma$ released upon activation of α_{2A}-R-$G\alpha_o$, in a similar way to transducin; as we have shown, however, $G\alpha_{qz5}$ is able to be activated. Once activated, it would then lead to stimulation of phospholipase C (Conklin et al., 1993), causing breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate and diacylglycerol, the latter stimulating PKC. Activation of PKC has been reported to counter G-protein modulation of rat $Ca_V2.2$ (Zamponi et al., 1997; Hamid et al., 1999). However, elevation of PIP_2 has also been shown to modulate $Ca_V2.1$, mimicking that by $G\beta\gamma$ (Wu et al., 2002). To investigate whether the reduction in inhibition and loss of facilitation in our coexpression studies with $G\alpha_{qz5}$ were caused by a $G\beta\gamma$ buffering effect or by a specific downstream effect of activated $G\alpha_{qz5}$ protein, we first chose to block the downstream action of activated $G\alpha_{qz5}$ by coexpressing the C-terminal peptide of phospholipase C-β1 (PLC-β1ct), which binds activated $G\alpha_q$ and acts as a GTPase-activating protein (Kammermeier and Ikeda, 1999). Inhibition by clonidine in cells coexpressing α_{2A}-R-$G\alpha_o$ and $G\alpha_{qz5}$ together with PLC-β1ct returned to levels comparable with when α_{2A}-R-$G\alpha_o$ was expressed alone (Fig. 6b). Furthermore the P2/P1 facilitation ratio in the presence of clonidine was increased relative to that in the presence of α_{2A}-R-$G\alpha_o$ and $G\alpha_{qz5}$ at all potentials between 0 and +20 mV, being 4.88 ± 2.48 at 0 mV and 2.25 ± 0.66 at +10 mV (Fig. 6d, $n = 6$, $p < 0.05$ relative to facilitation in clonidine for α_{2A}-R-$G\alpha_o$ and $G\alpha_{qz5}$ alone at 0 and +10 mV).

Because PLC-β1ct only binds activated G_q species and was able to reverse the effect of $G\alpha_{qz5}$, this must occur via its GTP-bound form. We therefore examined the role of downstream effectors of G_q. We investigated the effect of activating PKC to mimic the presence of $G\alpha_q$ as a signal transduction component, and simultaneously removed its presence as a potential $G\beta\gamma$ buffering agent. We used PDBu, an activator of PKC, on cells expressing the α_{2A}-R-$G\alpha_o$ fusion protein. After assessing the inhibition of $Ca_V2.2$ currents and the voltage-dependent facilitation elicited by clonidine alone, cells were perfused with PDBu (500 nM) in the presence of clonidine (Fig. 7, a and b). Within 5 min after the start of PDBu application, I_{Ba} partially recovered from inhibition by clonidine. During P1, inhibition by clonidine was reduced from 77.8 ± 6.1 to 56.1 ± 9.4% in the additional presence of PDBu (Fig. 7c, $n = 7$, $p < 0.001$). Application of PDBu also

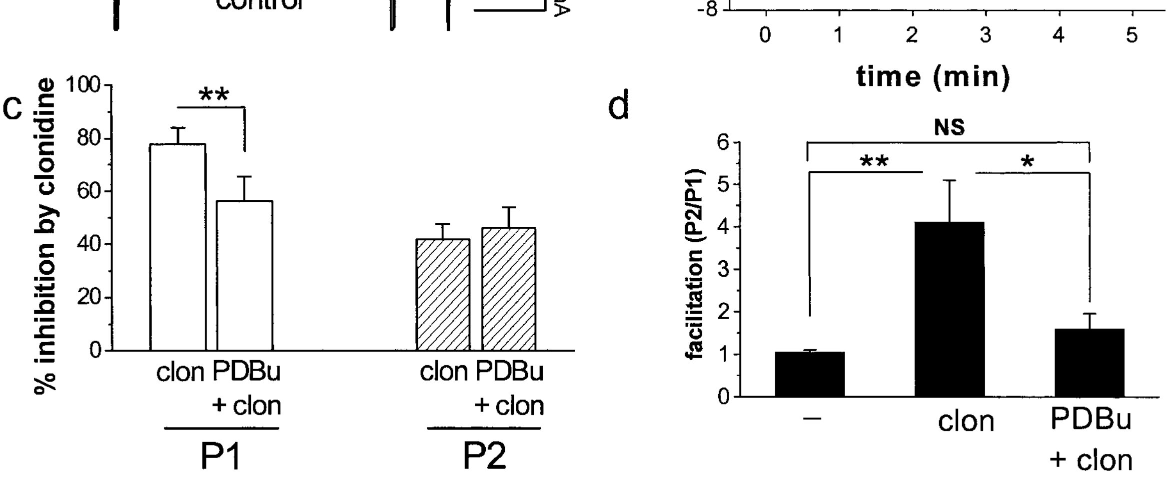

Fig. 7. Effect of an activator of PKC on clonidine-inhibited currents. a, superimposed example traces recorded from a cell expressing α_{2A}-R-$G\alpha_o$ during application of clonidine and after coapplication of clonidine and 500 nM PDBu. The voltage protocol used was that depicted in Fig. 1. b, time course from the same cell as in a for the current measured in P1 (●) and P2 (○). The letters correspond to the traces selected for a. c, percentage inhibition by clonidine before and during application of PDBu ($n = 7$), before (P1, □) and after (P2, ▨) the depolarizing PP. d, voltage-dependent facilitation for the same cells as in c in control (–), clonidine, and clonidine plus PDBu (statistical significances as indicated: *, $p < 0.05$; **, $p < 0.01$; NS, nonsignificant, paired *t* test).

resulted in reduced current facilitation (Fig. 7d, $n = 7$). After application of PDBu and clonidine, the current during P1 showed a rapid activation phase, further evidence for the loss of voltage-dependent inhibition (e.g., Fig. 7a, traces). Both the loss of inhibition and the reduction of facilitation are similar to the effect of $G\alpha_{qz5}$. Application of PDBu (500 nM) in the absence of receptor activation did not cause any increase of $Ca_V2.2$ I_{Ba}, rather reducing it by 37 ± 9% after application for 3 min, with a loss of control facilitation ($n = 6$, data not shown).

In a second approach to examine the involvement of PKC in the effects of $G\alpha_{qz5}$, we observed that the PKC inhibitor GF109203X partially restored the voltage-dependence of G-protein modulation in the presence of $G\alpha_{qz5}$. After a 30-min preincubation with 1 μM GF 109203X, application of clonidine to cells expressing α2A-R-$G\alpha_o$ and $G\alpha_{qz5}$ produced a 54 ± 15% inhibition of I_{Ba} at 0 mV ($n = 9$), and the P2/P1 facilitation ratio approached that in the absence of $G\alpha_{qz5}$ [2.4 ± 0.5 ($n = 9$)]. These two pieces of data indicate that PKC activation is at least in part responsible for the effects of $G\alpha_{qz5}$.

Discussion

The Advantage of Using GPCR-G Protein Tandems. We sought to recreate proximity between a GPCR, the α_{2A}R, and a specific G-protein by using tandem constructs. Both the chimeric receptors α_{2A}-R-$G\alpha_i$ and α_{2A}-R-$G\alpha_o$ reconstituted N-type current inhibition, comparable with the α_{2A}R w.t. Similarly, it has been found that a tandem between the muscarinic m2 receptor and $G\alpha_z$ was able to modulate GIRK channels by release of $G\beta\gamma$ (Vorobiov et al., 2000). This is in contrast to their inability to activate downstream effectors via the $G\alpha$ moiety (Sautel and Milligan, 1998; Burt et al., 1998), presumably because the $G\alpha$-subunits are not amplified and also because they are constrained. The conclusion of these results is that the release of $G\beta\gamma$ from both the activated GPCR tandems is completely sufficient to produce typical voltage-dependent inhibition of N-type calcium channels. This is confirmed by the inability of the IE mutant of α_{2A}-R-$G\alpha_o$, which does not bind $G\beta\gamma$, to mediate inhibition of $Ca_V2.2$ by clonidine. Although tonic facilitation was seen with this mutant in the absence of agonist (Fig. 3c), this was no greater than for the nontandem α_{2A}-R (Fig. 2c), where inhibition by clonidine was observed (Fig. 1e).

It has been proposed that members of the G_o subfamily are responsible for the voltage-dependent inhibition of calcium channels in sympathetic neurons, whereas G_i produced only a voltage-independent effect (Delmas et al., 1999). However, we did not find a clear correlation between the $G\alpha$-subunit in the tandem and the voltage-dependence of the inhibition, although there was a slightly greater voltage-dependent effect with the α_{2A}-R-$G\alpha_o$ fusion protein. This may relate to the endogenous $G\beta\gamma$ dimers with which the $G\alpha$ subunits preferentially associate. Indeed, the kinetics and voltage-dependence of $G\beta\gamma$ dissociation and reassociation are dependent on the nature of the $G\beta\gamma$ dimers (Stephens et al., 1998).

Effects of $G\alpha_q$ on G-Protein Modulation of Calcium Channels. The role of G_q in G-protein modulation of calcium currents remains unclear. It has been shown that G_q is not involved in modulation by the α_{2A}-R of the (largely N type) calcium currents in mouse sympathetic neurons (Haley et al., 2000). In the present study, expression of $G\alpha_q$ produced negligible inhibition of N-type channels, consistent with its very low ability to couple to the α_{2A}-R (Chabre et al., 1994). In contrast, the chimeric counterpart, $G\alpha_{qz5}$, allowed significant inhibition of $Ca_V2.2$, indicating that substitution of the C terminus of $G\alpha_z$ enhanced the coupling to the α_{2A}-R (Conklin et al., 1993). However, $G\alpha_{qz5}$ showed a reduced ability to inhibit I_{Ba} compared with $G_{i/o}$. The inhibition also showed a lack of voltage-dependence; together, these results suggested that G_{qz5} acts via a different or modified signaling mechanism compared with $G_{i/o}$. A similar voltage-independent inhibition of Ca^{2+} channels by the G_q-coupled muscarinic m1 receptor was shown to involve both the $G\alpha_q$ and $G\beta\gamma$ subunits (Kammermeier et al., 2000). Furthermore, the voltage-independent inhibition was converted into voltage-dependent inhibition by sequestering activated $G\alpha_q$ (Kammermeier and Ikeda, 1999).

In the present study, coexpression of $G\alpha_{qz5}$ with α_{2A}-R-$G\alpha_o$ caused first a reduction of clonidine-induced inhibition of $Ca_V2.2$ and second a loss of voltage-dependent facilitation. This action of $G\alpha_{qz5}$ could result from a number of mechanisms: 1) $G\beta\gamma$ buffering, as suggested for $G\alpha_q$ (Jeong and Ikeda, 1999), or 2) $G\alpha_{qz5}$ might interact with, and be activated by, the α_{2A}-R-$G\alpha_o$ tandem. It has been observed previously that chimeric receptor-$G\alpha$ constructs are able to activate not only the tethered $G\alpha$ subunit but also endogenous subunits of the $G_{i/o}$ family (Burt et al., 1998). In the case of $G\alpha_{qz5}$ this would result in downstream activation of phospholipase C, resulting in elevation of inositol 1,4,5-trisphosphate and diacylglycerol and concomitant reduction of PIP_2. One potential downstream pathway would be PKC activation and subsequent phosphorylation of either the calcium channel or the α_{2A}-R to suppress G-protein modulation. Another potential downstream pathway would be via reduction of PIP_2, because elevation of PIP_2 mimics and may play an essential role in G-protein modulation (Wu et al., 2002).

We have addressed these possibilities in turn. If the mechanism were $G\beta\gamma$ sequestration, $G\alpha_{qz5}$ should act identically to $G\alpha_t$. However $G\alpha_t$ reduced inhibition of $Ca_V2.2$ via α_{2A}-R-$G\alpha_o$ from 75 to 43% but did not abolish facilitation in the presence of clonidine (Fig. 3a, traces). In contrast, $G\alpha_{qz5}$ reduced inhibition by clonidine to 36% but completely abolished facilitation (Fig. 5a, traces). Furthermore, in cells coexpressing α_{2A}-R-$G\alpha_o$ and $G\alpha_{qz5}$, it was possible to restore typical G_o-mediated facilitation by enhancing the GTPase activity of activated $G\alpha_{qz5}$ with PLC-β1ct. This suggests that the effect of $G\alpha_{qz5}$ is downstream from its activation.

Two pieces of evidence indicate that PKC activation is involved in the response to $G\alpha_{qz5}$, although it may not represent the entire story. Firstly, application of a PKC agonist to cells expressing α_{2A}-R-$G\alpha_o$ mimicked the effects of $G\alpha_{qz5}$, resulting in reduced inhibition by clonidine and loss of PP facilitation. Secondly, in cells coexpressing α_{2A}-R-$G\alpha_o$ and $G\alpha_{qz5}$, G_o-mediated inhibition and facilitation were restored with a PKC inhibitor. Taken together, these results suggest that activation of the PLC-β signaling pathway by the α_{2A}-R coupling to G_{qz5} can oppose G-protein-mediated inhibition of $Ca_V2.2$.

Potential Targets for PKC Phosphorylation. PKC-mediated phosphorylation might occur at several sites, either separately or in combination. PKC activation has been shown to cause phosphorylation-dependent desensitization of the

α_{2A}-R in COS-7 and Chinese hamster ovary cells (Liang et al., 1998). It is possible that this process may play a role in the effect of $G\alpha_{qz5}$, although it is unclear how this could result in a selective loss of facilitation while substantial clonidine-mediated inhibition remains. Alternatively, PKC could phosphorylate one or more calcium channel subunits, thus rendering the channel less responsive to G-protein mediated inhibition and abolishing facilitation. There are a number of possible mechanisms by which this might occur. Phosphorylation of $\mathrm{Ca_V}2.2$ or an accessory β subunit may result in the loss of its ability to be modulated by $G\beta\gamma$. Residues in the I-II linker of rat $\mathrm{Ca_V}2.2$ have been proposed to be a target of phosphorylation by PKC and to be responsible for PKC antagonism of G-protein modulation (Zamponi et al., 1997). Evidence has been presented recently that this process involves binding of $G\alpha_q$ and PKC to the C terminus of $\mathrm{Ca_V}2.2$ (Simen et al., 2001). Direct activation of PKC has been shown to counteract inhibition of N-type calcium channels by norepinephrine (Shapiro et al., 1996). Subsequently, the importance was examined of phosphorylation sites on the I-II linker of rat $\mathrm{Ca_V}2.2$, including Thr^{422} and Ser^{425}, in the modulation by PKC (Zamponi et al., 1997). An increase in calcium current was observed when mimicking channel phosphorylation on either of these residues by mutation to Glu, and a reduction of G-protein modulation by somatostatin when Thr^{422} was mutated to Glu (Hamid et al., 1999). However, this effect was subsequently observed only with $G\beta1$ and not with other $G\beta$ subunits, calling into question its general relevance (Cooper et al., 2000). Indeed, we have shown that G-protein modulation of $\mathrm{Ca_V}2.2$ is not dependent on the presence of the I-II linker of a modulatable calcium channel, whereas the N terminus is essential (Canti et al., 1999).

Although the rabbit $\mathrm{Ca_V}2.2$ used in the present study shows very high overall sequence conservation in the I-II linker, and retains Ser^{425}, there is Ala at position 422, thus ruling out the role of phosphorylation of this residue in removing G-protein modulation. In addition, Ser^{425} is not an optimal consensus PKC phosphorylation motif (AAAKK**S**RSD). Furthermore, we did not observe any increase of N-type I_{Ba} upon application of PDBu in the absence of G-protein modulation. This would agree with results in superior cervical ganglion neurons, where the only effect of PKC activation was antagonism of G-protein inhibition (Barrett and Rittenhouse, 2000).

The mechanism of the reduction of G-protein modulation and loss of P2/P1 facilitation that is characteristic of coexpression of $G\alpha_{qz5}$ thus seems to involve its activation, and at least in part involves downstream activation of PKC, but the main target site(s) for phosphorylation may not be the calcium channel $\alpha1$ subunit. Facilitation involves the unbinding of $G\beta\gamma$ subunits from the channel during the depolarizing PP (Stephens et al., 1998); this requires the functional interaction of $\mathrm{Ca_V}\beta$ subunits (Canti et al., 2000, 2001; Meir et al., 2000). In the absence of coexpressed $\mathrm{Ca_V}\beta$ subunit, we observed previously that activation of the D2 dopamine receptor produced only a small voltage-independent inhibition of $\mathrm{Ca_V}2.2$ calcium channels, whereas in the presence of $\mathrm{Ca_V}\beta$ subunits, the inhibition was much larger and voltage-dependent (Meir et al., 2000). It is therefore possible that phosphorylation of the $\mathrm{Ca_V}\beta$ subunit might mediate the loss of facilitation resulting from $G\alpha_{qz5}$ coexpression. Indeed, another $\mathrm{Ca_V}\beta$ subunit, $\beta2a$, is phosphorylated stoichiometrically by PKC (Puri et al., 1997). We will examine in a future study whether phosphorylation of $\mathrm{Ca_V}\beta$ subunits by PKC is responsible for the effects of $G\alpha_{qz5}$.

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