

# 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  $\alpha_{2A}$  adrenergic receptor ( $\alpha_{2A}$ -R) and pertussis toxin-resistant mutants of different  $G\alpha$  subunits of heterotrimeric G-proteins. Both  $\alpha_{2A}$ -R- $G\alpha_o$  and  $\alpha_{2A}$ -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  $\alpha_{2A}$ -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  $\alpha_{2A}$ -R. It was found that  $G\alpha_{qz5}$  activated by the wild-type  $\alpha_{2A}$ -R inhibited  $Ca_v2.2$  currents in a voltage-independent fashion. Furthermore,

$G\alpha_{qz5}$  counteracted the voltage-dependent inhibition resulting from  $\alpha_{2A}$ -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  $C\beta1$ . 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  $\alpha_{2A}$ -R- $G\alpha_o$ -transfected cells. Conversely, cells expressing both  $\alpha_{2A}$ -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  $\alpha_{2A}$ -adrenergic receptor ( $\alpha_{2A}$ -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 se-

lective 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-

**ABBREVIATIONS:** GPCR, G-protein-coupled receptor;  $\alpha_{2A}$ -R,  $\alpha_{2A}$ -adrenergic receptor; PTX, pertussis toxin; GIRK, G-protein-coupled inward rectifier K channel; GFP, green fluorescent protein; w.t., wild type; RS-79948-197, [8aR,12aS,13aS]5,6,8a,9,10,11,12,12a,13,13a-decahydro-12-ethanesulfonyl-3-methoxy-6H-isoquinolo[2,1-g]-[1,6]naphthyridine hydrochloride; GTP $\gamma$ S, 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- $\beta1$ ct, phospholipase C- $\beta1$  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  $\alpha$  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  $\alpha_{2A}$ -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<sup>351</sup>  $G_{\alpha}$  mutants were chosen over other possible PTX-resistant mutants because they resulted in the strongest activation by  $\alpha_{2A}$ -R (Bahia et al., 1998). Activation of these tandems by the  $\alpha_{2A}$ -R agonist clonidine was studied in COS-7 cells coexpressing N-type channels (Ca<sub>v</sub>2.2) and comparing the response to that produced by the activation of the wild-type  $\alpha_{2A}$ -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<sup>2+</sup> 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  $\alpha_{2A}$ -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  $\alpha_{2A}$ -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<sub>v</sub>2.2 (GenBank accession no. D14157); rat  $\beta 1b$  (GenBank accession no. X11394); and mut-3 green fluorescent protein (GFP).

The PTX-resistant  $\alpha_{2A}$ -R-G-protein fusion proteins used throughout this study were prepared as described previously (Cavalli et al., 2000). In brief, Cys<sup>351</sup> of rat  $G_{\alpha_{i1}}$  and  $G_{\alpha_{o1}}$  was mutated to Ile by site-directed mutagenesis and then used to create the  $\alpha_{2A}$ -R- $G_{\alpha}$  fusion proteins using porcine  $\alpha_{2A}$ -R in pcDNA3. The Ile<sup>19</sup>Ala, Glu<sup>20</sup>Ala (IE) mutant of  $G_{\alpha_{o1}}$  was constructed, based on studies of an equivalent mutation (Ile<sup>25</sup>Ala, Glu<sup>26</sup>Ala) of  $G_{\alpha_q}$  (Evanko et al., 2000), and this was then incorporated into the PTX-resistant  $\alpha_{2A}$ -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- $\beta 1ct$  fusion construct of the C terminus of phospholipase C $\beta$  (PLC- $\beta 1ct$ ) 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  $\mu$ g/ml) (all from Invitrogen, Paisley, UK) at 37°C, 5% CO<sub>2</sub>, 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<sub>v</sub>2.2 subunit cDNA in a ratio of 3:1 with all the other constructs,  $\beta 1b$ ,  $\alpha_{2A}$ -R,  $\alpha_{2A}$ -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  $\mu$ l of GenePORTER reagent (Qbiogene, Harefield, UK) and 2  $\mu$ l 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  $2 \times 10^6$  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  $G_{i/o}$  subunits by adding it to the culture medium at a concentration of 40 to 100 ng/ml for 16 h before replating the cells.

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

**[<sup>35</sup>S]GTP $\gamma$ S Binding.** [<sup>35</sup>S]GTP $\gamma$ S binding experiments were performed essentially as described for receptor-G-protein tandems incorporating  $G_{\alpha_{i1}}$  (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<sub>2</sub>, 100 mM NaCl, 1  $\mu$ M guanosine 5'-diphosphate, 0.2 mM ascorbic acid, and 50 nCi of [<sup>35</sup>S]GTP $\gamma$ S] in the absence or presence of clonidine (10  $\mu$ M). Non-specific binding was determined in the same conditions but in the presence of 100  $\mu$ M GTP $\gamma$ S. 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<sub>2</sub>, and 100 mM NaCl. The samples were centrifuged at 16,000g 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  $\alpha_{2A}$ -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 [<sup>35</sup>S]GTP $\gamma$ S was measured by liquid scintillation counting.

**Immunoprecipitation and Immunodetection Studies.** To analyze the interaction of  $\alpha_{2A}$ -R- $G_{\alpha_o}$  with  $G\beta\gamma$  dimers, cells were transfected with  $\alpha_{2A}$ -R- $G_{\alpha_o}$  or  $\alpha_{2A}$ -R-Ile<sup>19</sup>Ala, Glu<sup>20</sup>Ala  $G_{\alpha_o}$  in the absence or presence of plasmids encoding G-protein  $\beta 1$  and  $\gamma 2$  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<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 100 mM NaF, 10 mM EDTA, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 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 immunoblotting, 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  $\beta 1$  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.

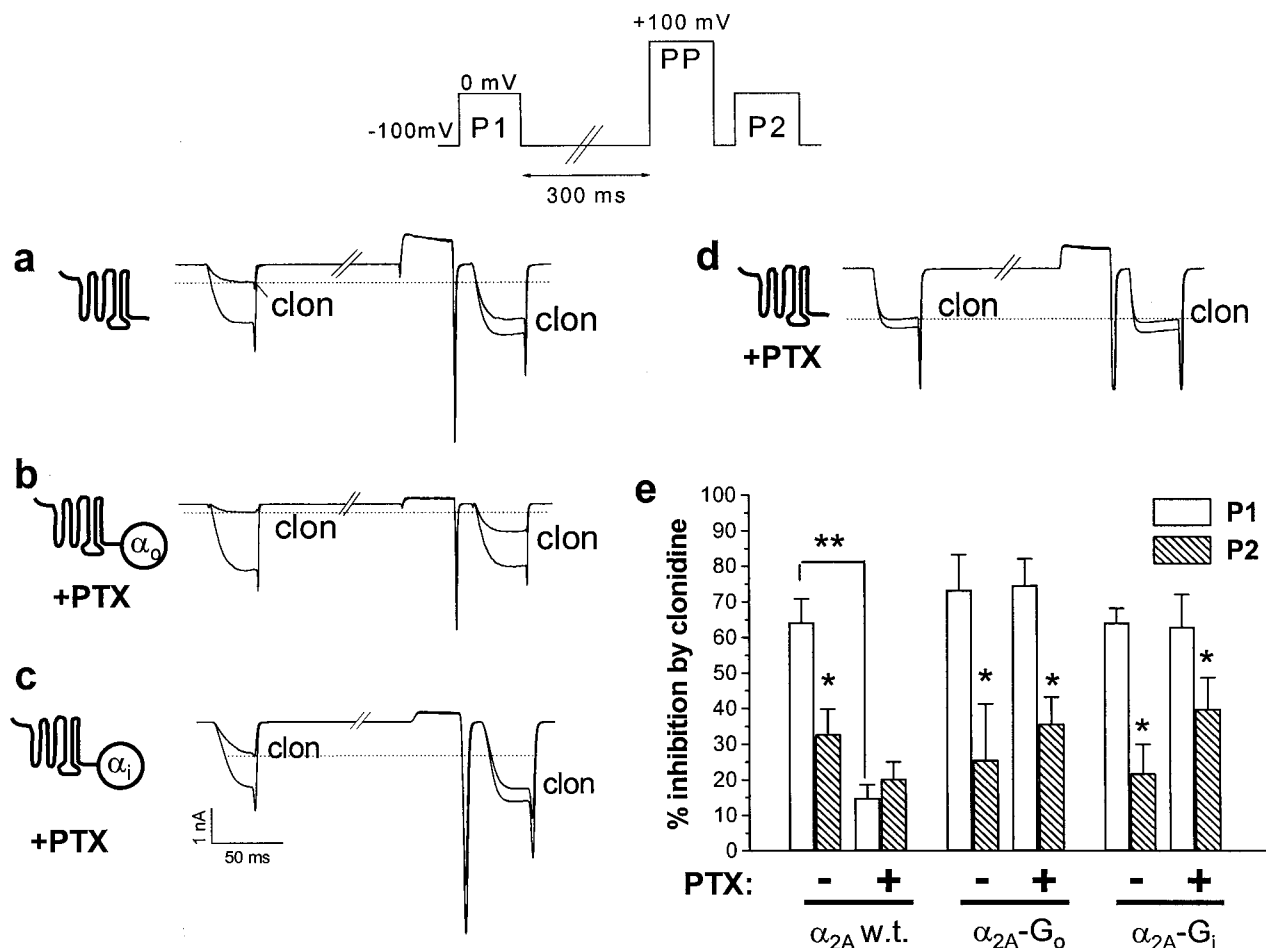
**Electrophysiology.** Fluorescent COS-7 cells expressing GFP were chosen for whole-cell, patch-clamp recording. Borosilicate glass electrodes were used with a resistance of 2 to 5 M $\Omega$  when filled with a solution containing 140 mM cesium aspartate, 5 mM EGTA, 2 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 2 mM K<sub>2</sub>ATP, 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<sub>3</sub>, 1.0 MgCl<sub>2</sub>, 10 mM HEPES, 4 mM glucose, and 10 mM BaCl<sub>2</sub>, 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 ( $20$ – $24^{\circ}\text{C}$ ). 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:  $I = G_{\text{max}} (V - V_{\text{rev}}) / (1 + \exp(-(V - V_{50,\text{act}})/k))$ , where  $I$  is the current density (picoamperes per picofarad),  $G_{\text{max}}$  is the maximal conductance (nanosiemens per picofarad),  $V_{\text{rev}}$  is the reversal potential,  $V_{50,\text{act}}$  is the mid-point voltage for current activation, and  $k$  is the slope factor.

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

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



**Fig. 1.** Comparison of inhibition of  $I_{\text{Ba}}$  by  $\alpha_{2A}$ -R and  $\alpha_{2A}$ -R- $G_{i/o}$  tandems. Top, double-pulse voltage-clamp protocol used to measure the PP facilitation of  $I_{\text{Ba}}$ . 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  $\alpha_{2A}$ -R constructs is given on the left. Example recording from cells expressing different receptor constructs. Currents recorded in control and after application of  $10 \mu\text{M}$  clonidine are superimposed. a, the  $\alpha_{2A}$ -R w.t.; b, the PTX-resistant  $\alpha_{2A}$ -R- $G_{i/o}$ ; c, the PTX-resistant  $\alpha_{2A}$ -R- $G_{i/o}$ ; d, example traces after preincubation with PTX from a cell expressing  $\alpha_{2A}$ -R w.t.; e, summary of  $I_{\text{Ba}}$  inhibition by clonidine before (P1,  $\square$ ) and after (P2,  $\blacksquare$ ) the depolarizing PP. Values are reported without and with pretreatment with PTX for the  $\alpha_{2A}$ -R w.t. ( $n = 4$  and  $9$ , respectively), and for the receptor tandems  $\alpha_{2A}$ -R- $G_{i/o}$  ( $n = 5$  and  $18$ , respectively) and  $\alpha_{2A}$ -R- $G_{i/o}$  ( $n = 8$  for both) (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; either paired  $t$  test, between P1 and P2, or unpaired  $t$  test between  $\pm$  PTX, as indicated).



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

## Results

**Effect of the  $\alpha_{2A}$  Adrenergic Receptor- $G\alpha_i$  and - $G\alpha_o$  Tandems.** We first expressed either  $\alpha_{2A}$ -R w.t. or the PTX-insensitive receptor- $G\alpha$  tandems  $\alpha_{2A}$ -R- $G\alpha_{o1}C^{351}I$  ( $\alpha_{2A}$ -R- $G\alpha_o$ ) or  $\alpha_{2A}$ -R- $G\alpha_iC^{351}I$  ( $\alpha_{2A}$ -R- $G\alpha_i$ ) together with the Ca<sub>v</sub>2.2 calcium channel. The inhibition of the expressed Ba<sup>2+</sup> currents ( $I_{Ba}$ ) by activation of the  $\alpha_{2A}$ -R w.t. was compared with the effect of the receptor G-protein tandems (Fig. 1). Overall, the  $\alpha_{2A}$ -R agonist clonidine (10  $\mu$ M) inhibited N-type  $I_{Ba}$  via activation of both the free  $\alpha_{2A}$ -R and the tandem  $\alpha_{2A}$ -R- $G\alpha$  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 ( $\square$ ). In the absence of PTX,  $I_{Ba}$  was similarly reduced by both the wild-type  $\alpha_{2A}$ -R ( $64.2 \pm 6.6\%$ ,  $n = 9$ , Fig. 1a) and the tandems  $\alpha_{2A}$ -R- $G\alpha_o$  ( $77.6 \pm 6.6\%$ ,  $n = 5$ , Fig. 1b) and  $\alpha_{2A}$ -R- $G\alpha_i$  ( $64.1 \pm 4.0\%$ ,  $n = 8$ , 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<sub>v</sub>2.2  $I_{Ba}$ .

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). The use of PTX therefore allows isolation of the effects of exogenous  $G\alpha$  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  $\alpha_{2A}$ -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  $74.1 \pm 6.5\%$  ( $n = 18$ , Fig. 1b) and  $62.9 \pm 9.1\%$  ( $n = 8$ , Fig. 1c) with the  $G\alpha_o$  and the  $G\alpha_i$  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  $\alpha_{2A}$ -R w.t. (Fig. 1a) or the  $\alpha_{2A}$ -R- $G\alpha_o$  (Fig. 1b) and  $\alpha_{2A}$ -R- $G\alpha_i$  (Fig. 1c) tandems, whereas incubation with PTX eliminated the voltage-dependent effects of the  $\alpha_{2A}$ -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  $\alpha_{2A}$ -R- $G\alpha_o$  ( $n = 8$ ) 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  $\alpha_{2A}$ -R- $G\alpha_o$ , from  $-6.4 \pm 3.1$  to  $+9.0 \pm 4.0$  mV ( $p < 0.05$ ,  $n = 6$ , Fig. 2b) and, for cells expressing  $\alpha_{2A}$ -R- $G\alpha_i$ , from  $+2.5 \pm 2.4$  to  $+9.7 \pm 0.7$  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 ( $\square$ ), which was more marked when expressing the  $\alpha_{2A}$ -R w.t., where P2/P1 was  $2.3 \pm 0.3$  at 0 mV (Fig. 2c). Clonidine enhanced the voltage-dependent facilitation, although the effects were much greater for the  $\alpha_{2A}$ -R- $G\alpha_o$  tandem than for the  $\alpha_{2A}$ -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  $\alpha_{2A}$ -R- $G\alpha$  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  $\alpha_{2A}$ -R- $G\alpha_o$  tandem, the  $\tau_{act}$  at 0 mV during P1 was  $3.7 \pm 0.5$  ms in control and  $6.1 \pm 1.1$  ms during clonidine application ( $n = 10$ ,  $p < 0.05$ , see Fig. 1b). This slowed activation was reversed by  $G\alpha_t$ , which acts as a  $G\beta\gamma$  sink to sequester free  $G\beta\gamma$  subunits but does not couple to the  $\alpha_{2A}$ -R. Example traces are shown in Fig. 3a (top). After cotransfection of  $G\alpha_t$  with  $\alpha_{2A}$ -R- $G\alpha_o$ , there was no longer a difference in the  $\tau_{act}$  values measured in control and clonidine during P1 ( $2.9 \pm 0.6$  ms and  $3.4 \pm 0.5$  ms, respectively,  $n = 9$ ). Along with this effect,  $G\alpha_t$  was able significantly to reduce inhibition by clonidine at 0 mV from  $74.1 \pm 6.5$  to  $43.0 \pm 6.7\%$  ( $p < 0.001$ ; Fig. 3b) and to reduce the P2/P1 facilitation ratio in the presence of clonidine to  $1.54 \pm 0.24$  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  $G_{i/o}$  proteins, these findings indicate that the  $\alpha_{2A}$ -R tandems are able to reconstitute inhibitory effects on Ca<sub>v</sub>2.2 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<sup>25</sup> and Glu<sup>26</sup> 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  $\alpha$  subunits. We thus constructed a form of the PTX-resistant  $\alpha_{2A}$ -R- $G\alpha_o$  tandem (IE) that also incorporated the equivalent mutations of Ile<sup>19</sup>Ala and Glu<sup>20</sup>Ala in  $G\alpha_o$ . Application of clonidine to cells expressing the IE form of the  $\alpha_{2A}$ -R- $G\alpha_o$  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<sub>v</sub>2.2 currents show some tonic modulation, being slowly activating and facilitated by a pre-

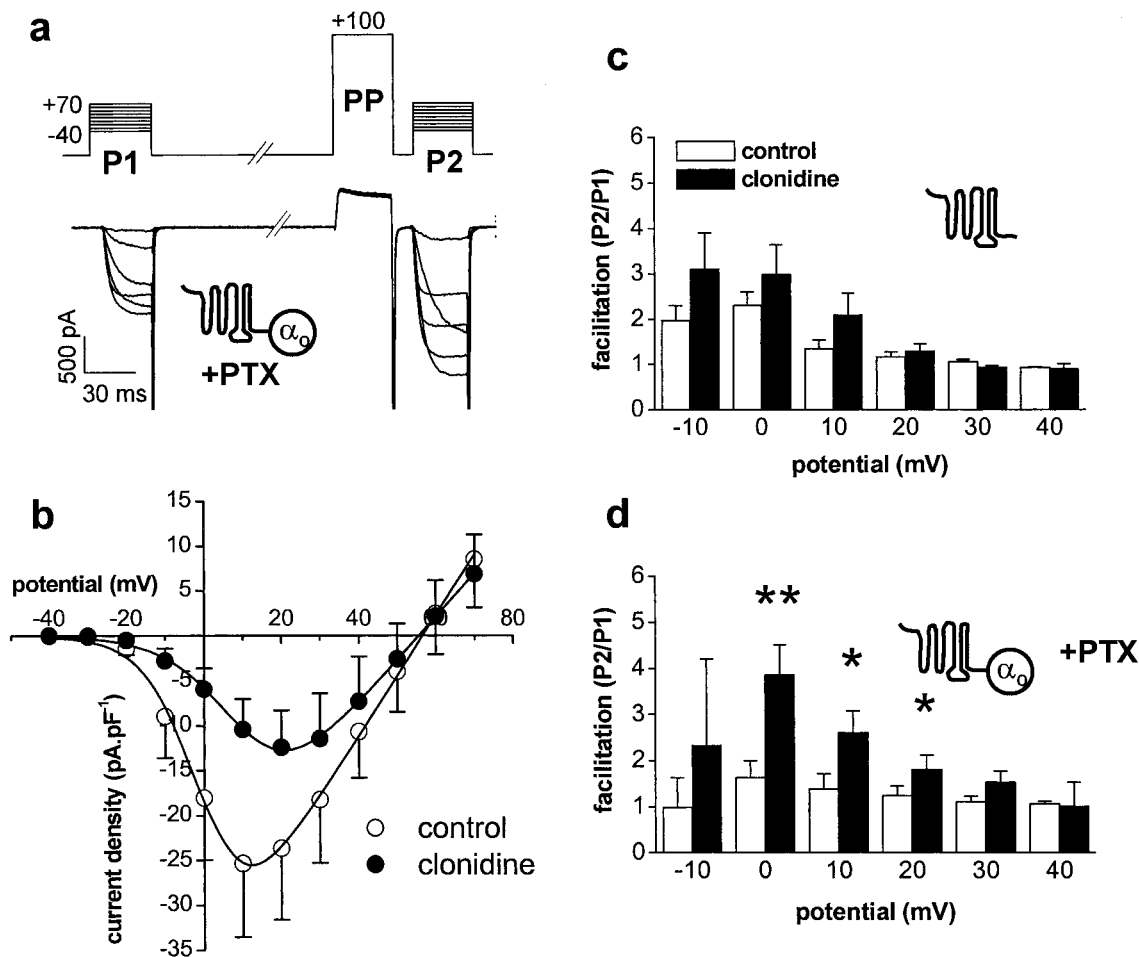
pulse, although this is no greater than for the free  $\alpha_{2A}$ -R (Fig. 2c).

To examine the binding of  $G\beta\gamma$  to the IE mutant of  $\alpha_{2A}$ -R- $G\alpha_o$ , either  $\alpha_{2A}$ -R- $G\alpha_o$  or the IE form of this construct was cotransfected together with plasmids encoding the  $G\beta 1$  and  $G\gamma 2$  subunits. Cell lysates were subsequently immunoprecipitated with an antiserum (OC) that identifies the C-terminal decapeptide of  $G\alpha_{o1}$ . 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  $G\beta 1$ . Although the  $\alpha_{2A}$ -R- $G\alpha_o$  tandem allowed coimmunoprecipitation of  $\beta 1$  subunit (Fig. 3d, lane 2), this was not observed for the IE form of the tandem receptor (Fig. 3d, lane 3).

**Investigation of  $\alpha_{2A}$ -R- $G\alpha_q$  and  $\alpha_{2A}$ -R- $G\alpha_{qz5}$  Chimeras.** Because the expression of the receptor/G-protein tandems indicated that the release of activated  $G\alpha$  subunits,  $G\alpha_i$  and  $G\alpha_o$ , 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  $\alpha_{2A}$ -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  $G\alpha_{qz5}$ . This subunit conserved the main structure of  $G\alpha_q$  but the last five amino acids were substituted for those of  $G\alpha_z$ , a PTX-resistant member of the  $G_{i/o}$  family that does couple to the  $\alpha_{2A}$ -R (Conklin et al., 1993). Tandem  $\alpha_{2A}$ -R- $G\alpha_q$  and  $\alpha_{2A}$ -R- $G\alpha_{qz5}$  constructs were assembled, and their functionality was assessed biochemically.

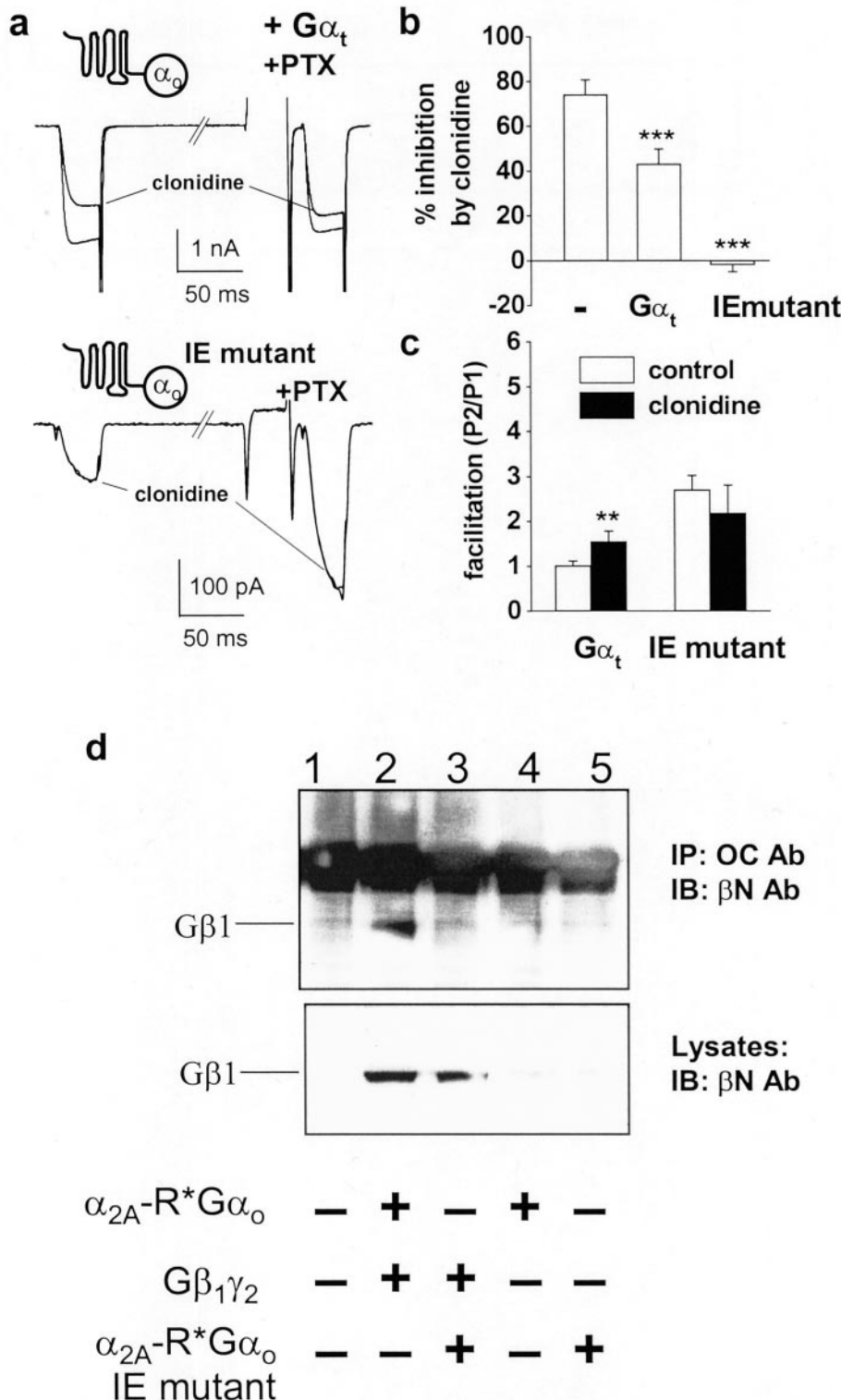
Evidence of the activation of the PTX-resistant G-proteins within the  $\alpha_{2A}$ -R- $G\alpha$  tandems by clonidine was obtained by monitoring agonist-induced binding of [ $^{35}$ S]GTP $\gamma$ S. Expression levels of the  $\alpha_{2A}$ -R-containing fusion proteins in membranes of PTX-treated cells were quantified by saturation ligand binding studies employing the high-affinity  $\alpha_2$ -adrenoceptor antagonist [ $^3$ H]RS-79948-197. [ $^{35}$ S]GTP $\gamma$ S binding studies were performed in the presence and absence of clonidine (10  $\mu$ M) 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]GTP $\gamma$ S binding were observed for both the  $G\alpha_o$ - and  $G\alpha_i$ -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  $\alpha_{2A}$ -R- $G\alpha_o$  and treated with PTX. **b**, I-V relationship for cells expressing  $\alpha_{2A}$ -R- $G\alpha_o$  before the PP. The data are average values of current density before ( $\circ$ ) and after ( $\bullet$ ) 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 ( $\square$ ) and in the presence of clonidine ( $\blacksquare$ ), for the  $\alpha_{2A}$ -R w.t. in the absence of PTX ( $n = 4$ ) (**c**) and  $\alpha_{2A}$ -R- $G\alpha_o$  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 [<sup>35</sup>S]GTPγS was observed to the α<sub>2A</sub>-R-Gα<sub>q</sub> and α<sub>2A</sub>-R-Gα<sub>qz5</sub> constructs, even in the presence of clonidine, consistent with a lack of activation of these G-proteins by the associated α<sub>2A</sub>-R. The inability of clonidine to promote binding of [<sup>35</sup>S]GTPγS to the fusion proteins containing Gα<sub>q</sub> does not reflect the well appreciated difficulty in monitoring nucleotide

exchange for such G-proteins in standard [<sup>35</sup>S]GTPγS 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<sub>v</sub>2.2 via the α<sub>2A</sub>-R-Gα<sub>qz5</sub> tandem, but because



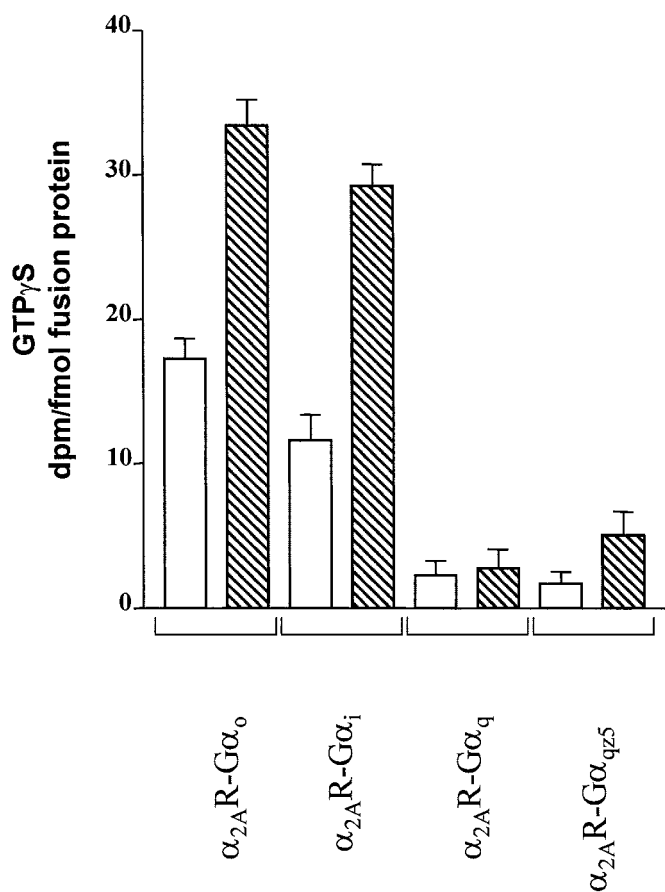
**Fig. 3.** The effects of α<sub>2A</sub>-R-Gα<sub>o</sub> are mediated by Gβγ. **A**, example traces recorded in cells expressing the α<sub>2A</sub>-R-Gα<sub>o</sub> tandem and Gα<sub>t</sub> (upper traces) or the IE mutant of α<sub>2A</sub>-R-Gα<sub>o</sub> (lower traces). **b**, inhibition by clonidine in cells expressing α<sub>2A</sub>-R-Gα<sub>o</sub> alone (*n* = 10), α<sub>2A</sub>-R-Gα<sub>o</sub>, and Gα<sub>t</sub> (*n* = 9) or the IE mutant of α<sub>2A</sub>-R-Gα<sub>o</sub> (*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<sup>19</sup> and Glu<sup>20</sup> of Gα<sub>o</sub> inhibits interaction with the G-protein β1 subunit. Cells were mock transfected (lane 1) or transfected with either the α<sub>2A</sub>-R-Gα<sub>o</sub> fusion protein (lanes 2, 4) or the α<sub>2A</sub>-R-(Ile<sup>19</sup>Ala, Glu<sup>20</sup>Ala)-Gα<sub>o</sub> fusion (IE mutant, lanes 3 and 5). In lanes 2 and 3, cells were also transfected with plasmids encoding Gβ1 and Gγ2. Top, samples were immunoprecipitated with antiserum OC against the C-terminal of Gα<sub>o</sub>, 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 β1 subunit. Data are from a representative experiment.

these receptor- $G\alpha_q$  tandems were nonfunctional biochemically, their coupling to  $Ca_v2.2$  was not further examined.

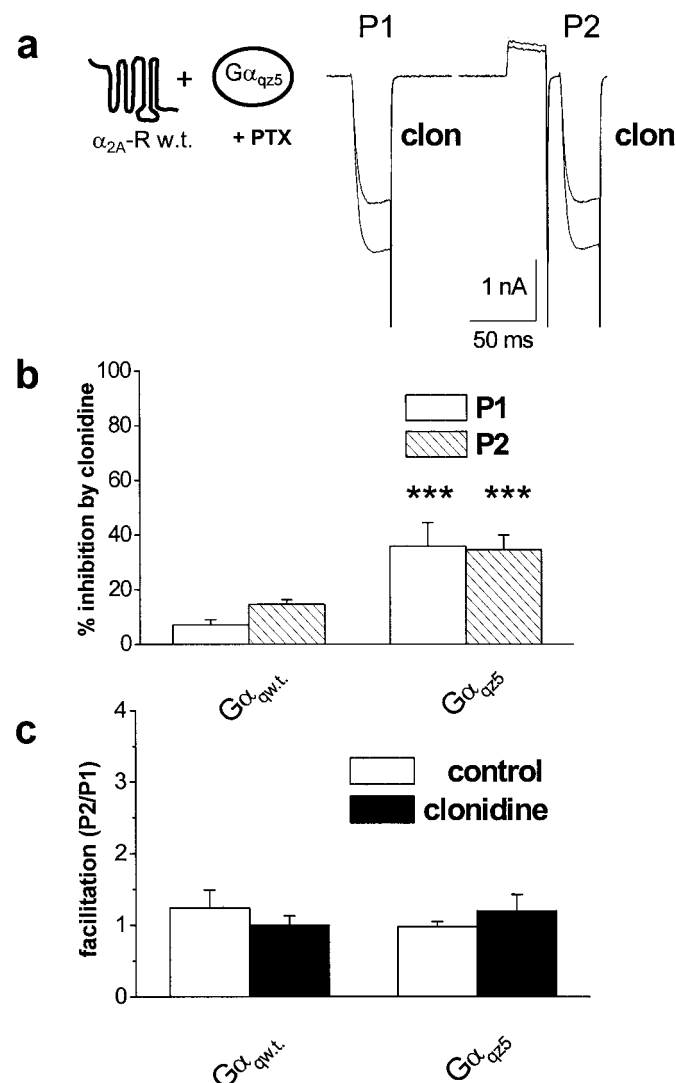
We therefore employed free  $G\alpha_q$  and  $G\alpha_{qz5}$  to examine whether  $G\beta\gamma$  released from  $G_q$  or  $G_{qz5}$  can signal to N-type calcium channels (Fig. 5a). We confirmed, by coexpressing the  $\alpha_{2A}$ -R w.t. with  $G\alpha_q$  w.t. in cells treated with PTX, that  $G\alpha_q$  did not couple directly to the  $\alpha_{2A}$ -R. Perfusion of clonidine induced only  $7.1 \pm 1.1\%$  reduction in the current ( $n = 5$ , Fig. 5b). In contrast, expression of  $G\alpha_{qz5}$  with the  $\alpha_{2A}$ -R w.t. resulted in significantly greater inhibition of  $Ca_v2.2$  currents by clonidine ( $35.8 \pm 8.6\%$ ,  $n = 9$ , Fig. 5, a and b). Surprisingly however, this was not removed by a PP to +100 mV, the inhibition in P2 being  $34.5 \pm 5.4\%$  ( $n = 9$ , Fig. 5b). Thus, the inhibition elicited by  $G\alpha_{qz5}$  was much greater than that elicited by  $G\alpha_q$  w.t. ( $p < 0.001$ ) but was not voltage-dependent. The P2/P1 facilitation ratio in the presence of  $G\alpha_{qz5}$  was around unity and was unaffected by the presence of agonist ( $0.98 \pm 0.07$  in control,  $1.20 \pm 0.23$  in clonidine,  $p > 0.05$ , Fig. 5c). Current traces in the presence of  $G\alpha_{qz5}$  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  $G\alpha_{qz5}$ , we also examined the voltage-dependence of inhibition over a range of poten-

tials. 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  $G\alpha_{qz5}$  to couple to the  $\alpha_{2A}$ -R, causing a reduction in  $I_{Ba}$ , although the inhibition was voltage-independent and smaller than that elicited by the tandems  $\alpha_{2A}$ -R- $G\alpha_o$  and  $\alpha_{2A}$ -R- $G\alpha_i$  or the wild type  $\alpha_{2A}$ -R coupling to endogenous G-proteins.

**Interaction between  $\alpha_{2A}$ -R- $G\alpha_o$  and  $G\alpha_{qz5}$ .** 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). Accordingly,  $G\alpha_{qz5}$  might be expected also to interact with, and to be activated by, the  $\alpha_{2A}$ -R- $G\alpha_o$  tandem used in this part of the study. We investigated this potential interaction by coexpressing the tandem  $\alpha_{2A}$ -R- $G\alpha_o$  with the  $G\alpha_{qz5}$  subunit, and treating all cells with PTX.



**Fig. 4.** Clonidine stimulates binding of [ $^{35}$ S]GTP $\gamma$ S to fusion proteins between the  $\alpha_{2A}$ -R and both  $G\alpha_{o1}$  and  $G\alpha_{i1}$ . Membranes were prepared from cells transfected to express fusion proteins between an N-terminally HA-tagged form of the  $\alpha_{2A}$ -R and each of (Cys $^{351}$ Ile)  $G\alpha_o$ , (Cys $^{351}$ Ile)  $G\alpha_i$ ,  $G\alpha_q$ , or  $G\alpha_{qz5}$ . After [ $^{35}$ S]GTP $\gamma$ S binding assays performed in the absence (□) or presence (▨) of clonidine (10  $\mu$ M), samples were immunoprecipitated with the anti-HA antibody 12CA5 and  $^{35}$ S content was determined. Data represent mean  $\pm$  S.E.M. ( $n = 3$ ).

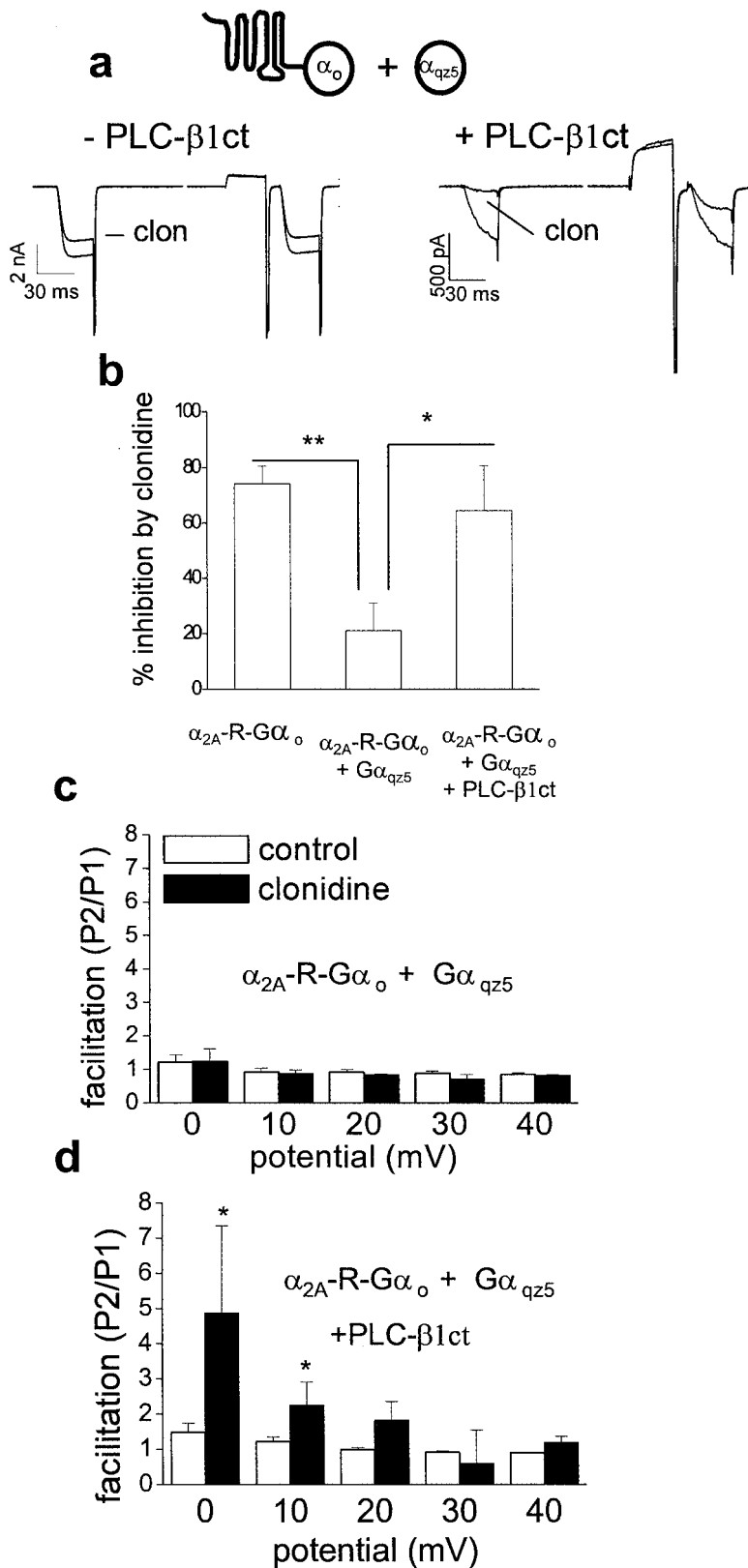


**Fig. 5.** The ability of the  $G\alpha_{qz5}$  subunit to support G-protein modulation of  $Ca_v2.2$  currents. a, example traces recorded in the presence and absence of clonidine from a cell expressing the  $\alpha_{2A}$ -R w.t. and  $G\alpha_{qz5}$ . b, inhibition by clonidine in cells expressing the  $\alpha_{2A}$ -R w.t. with the  $G\alpha_q$  w.t. subunit ( $n = 5$ , left) or the  $\alpha_{2A}$ -R w.t. with the  $G\alpha_{qz5}$  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  $G\alpha_q$  w.t., \*\*\*,  $p < 0.001$ , Student's  $t$  test.



The first observation was that the inhibition of  $I_{Ba}$  obtained when coexpressing  $\alpha_{2A}$ -R-G $\alpha_o$  with G $\alpha_{qz5}$  was significantly smaller than in cells expressing  $\alpha_{2A}$ -R-G $\alpha_o$  alone (Fig. 6a). Inhibition was  $21.0 \pm 12.4\%$  in P1 ( $n = 16$ ,  $p < 0.01$ , Fig. 6b). Interestingly, the presence of G $\alpha_{qz5}$  also almost abol-

ished facilitation by the PP at all potentials examined (Fig. 6c). For example the P2/P1 facilitation ratio in clonidine was  $1.24 \pm 0.38$  at 0 mV and  $0.87 \pm 0.11$  at +10 mV ( $n = 11$ , both  $p < 0.01$  compared with the much greater facilitation shown by the  $\alpha_{2A}$ -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- $\beta$ 1. **a**, example recordings from cells expressing both the tandem  $\alpha_{2A}$ -R-G $\alpha_o$  and the G $\alpha_{qz5}$  subunit without (left) and with (right) the additional presence of PLC- $\beta$ 1ct. The voltage protocol is that shown in Fig. 1. **b**, mean percentage inhibition by clonidine for  $\alpha_{2A}$ -R-G $\alpha_o$  alone ( $n = 18$ );  $\alpha_{2A}$ -R-G $\alpha_o$  and G $\alpha_{qz5}$  ( $n = 16$ ); and  $\alpha_{2A}$ -R-G $\alpha_o$ , G $\alpha_{qz5}$ , and PLC- $\beta$ 1ct ( $n = 6$ ). Statistical significance, \*\*,  $p < 0.01$ ; \*,  $p < 0.05$  as indicated. **c**, voltage-dependence of facilitation ratio for coexpression of  $\alpha_{2A}$ -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  $\alpha_{2A}$ -R-G $\alpha_o$ , G $\alpha_{qz5}$ , and PLC- $\beta$ 1ct ( $n = 6$ ). Voltage protocol as in Fig. 2a. Statistical significance, \*,  $p < 0.05$ , compared with the P2/P1 ratio in clonidine for  $\alpha_{2A}$ -R-G $\alpha_o$ , G $\alpha_{qz5}$  in the absence of PLC- $\beta$ 1ct (given in Fig. 6c).

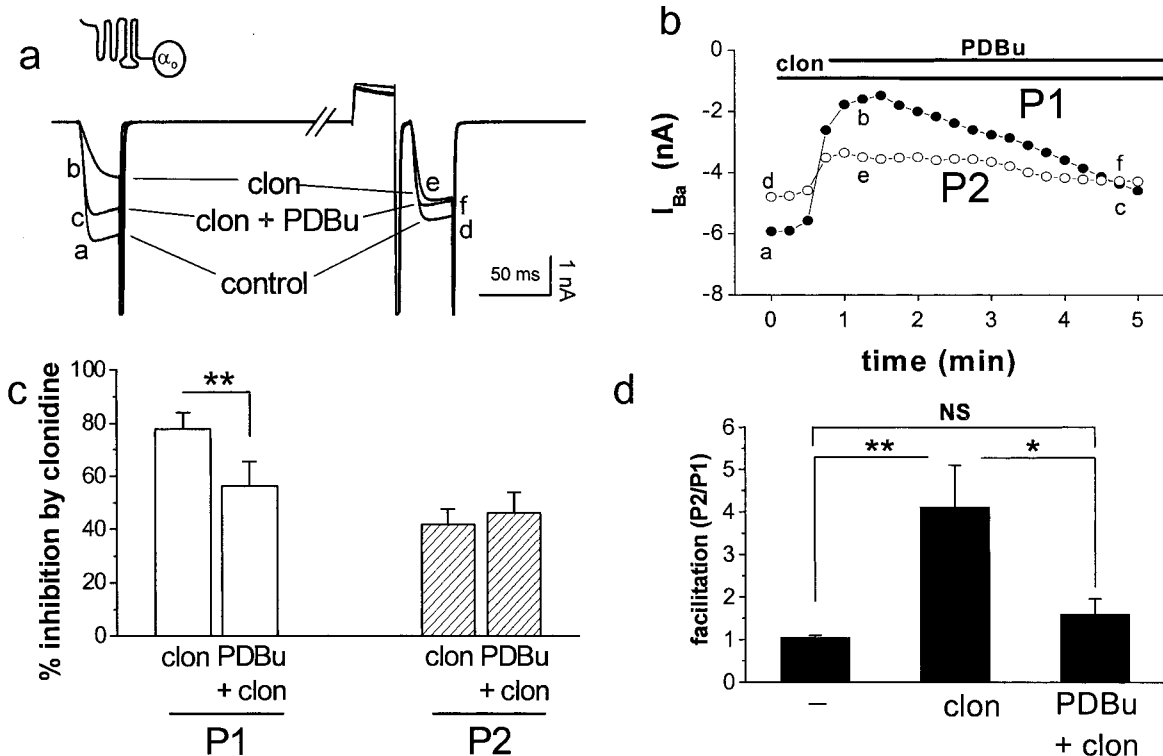


induced depolarizing shift of the I-V relationship for  $I_{Ba}$  was detected (data not shown). Furthermore, no slowing of the activation kinetics was evident during P1 (e.g., Fig. 6a). In summary, coexpression of  $G\alpha_{qz5}$  with  $\alpha_{2A}$ -R- $G\alpha_o$  reduced the inhibition and reversed the P2/P1 facilitation observed upon activation of the  $\alpha_{2A}$ -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  $\alpha_{2A}$ -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- $\beta 1$  (PLC- $\beta 1$ ct), which binds activated  $G\alpha_q$  and acts as a GTPase-activating protein (Kammermeier and Ikeda, 1999). Inhibition by clonidine in cells coexpressing  $\alpha_{2A}$ -R- $G\alpha_o$  and  $G\alpha_{qz5}$  together with PLC- $\beta 1$ ct returned to levels comparable with when  $\alpha_{2A}$ -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  $\alpha_{2A}$ -R- $G\alpha_o$  and  $G\alpha_{qz5}$  at all potentials between 0 and +20 mV, being  $4.88 \pm 2.48$  at 0 mV and  $2.25 \pm 0.66$  at +10 mV (Fig. 6d,  $n = 6$ ,  $p < 0.05$  relative to facilitation in clonidine for  $\alpha_{2A}$ -R- $G\alpha_o$  and  $G\alpha_{qz5}$  alone at 0 and +10 mV).

Because PLC- $\beta 1$ ct 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  $\alpha_{2A}$ -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  $77.8 \pm 6.1$  to  $56.1 \pm 9.4\%$  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  $\alpha_{2A}$ -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<sub>v</sub>2.2  $I_{Ba}$ , rather reducing it by  $37 \pm 9\%$  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  $\mu$ M GF 109203X, application of clonidine to cells expressing  $\alpha_{2A}$ -R- $G_{\alpha_o}$  and  $G_{\alpha_{qz5}}$  produced a  $54 \pm 15\%$  inhibition of  $I_{Ba}$  at 0 mV ( $n = 9$ ), and the P2/P1 facilitation ratio approached that in the absence of  $G_{\alpha_{qz5}}$  [ $2.4 \pm 0.5$  ( $n = 9$ )]. 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  $\alpha_{2A}$ R, and a specific G-protein by using tandem constructs. Both the chimeric receptors  $\alpha_{2A}$ -R- $G_{\alpha_i}$  and  $\alpha_{2A}$ -R- $G_{\alpha_o}$  reconstituted N-type current inhibition, comparable with the  $\alpha_{2A}$ 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  $\alpha_{2A}$ -R- $G_{\alpha_o}$ , which does not bind  $G\beta\gamma$ , to mediate inhibition of Ca<sub>v</sub>2.2 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  $\alpha_{2A}$ -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  $\alpha_{2A}$ -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  $\alpha_{2A}$ -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  $\alpha_{2A}$ -R (Chabre et al., 1994). In contrast, the chimeric counterpart,  $G_{\alpha_{qz5}}$ , allowed significant inhibition of Ca<sub>v</sub>2.2, indicating that substitution of the C terminus of  $G_{\alpha_z}$  enhanced the coupling to the  $\alpha_{2A}$ -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  $G_{\alpha_{qz5}}$  acts via a different or modified signaling mechanism compared with  $G_{i/o}$ . A similar voltage-independent inhibition of Ca<sup>2+</sup> 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  $\alpha_{2A}$ -R- $G_{\alpha_o}$  caused first a reduction of clonidine-induced inhibition of Ca<sub>v</sub>2.2 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  $\alpha_{2A}$ -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<sub>2</sub>. One potential downstream pathway would be PKC activation and subsequent phosphorylation of either the calcium channel or the  $\alpha_{2A}$ -R to suppress G-protein modulation. Another potential downstream pathway would be via reduction of PIP<sub>2</sub>, because elevation of PIP<sub>2</sub> 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<sub>v</sub>2.2 via  $\alpha_{2A}$ -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  $\alpha_{2A}$ -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- $\beta$ 1ct. 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  $\alpha_{2A}$ -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  $\alpha_{2A}$ -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- $\beta$  signaling pathway by the  $\alpha_{2A}$ -R coupling to  $G_{\alpha_{qz5}}$  can oppose G-protein-mediated inhibition of Ca<sub>v</sub>2.2.

**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

$\alpha_{2A}$ -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  $Ca_v2.2$  or an accessory  $\beta$  subunit may result in the loss of its ability to be modulated by  $G\beta\gamma$ . Residues in the I-II linker of rat  $Ca_v2.2$  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  $Ca_v2.2$  (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  $Ca_v2.2$ , including Thr<sup>422</sup> and Ser<sup>425</sup>, 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<sup>422</sup> was mutated to Glu (Hamid et al., 1999). However, this effect was subsequently observed only with  $G\beta 1$  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  $Ca_v2.2$  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  $Ca_v2.2$  used in the present study shows very high overall sequence conservation in the I-II linker, and retains Ser<sup>425</sup>, there is Ala at position 422, thus ruling out the role of phosphorylation of this residue in removing G-protein modulation. In addition, Ser<sup>425</sup> is not an optimal consensus PKC phosphorylation motif (AAAKKSRSD). 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  $\alpha 1$  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  $Ca_v\beta$  subunits (Canti et al., 2000, 2001; Meir et al., 2000). In the absence of coexpressed  $Ca_v\beta$  subunit, we observed previously that activation of the D2 dopamine receptor produced only a small voltage-independent inhibition of  $Ca_v2.2$  calcium channels, whereas in the presence of  $Ca_v\beta$  subunits, the inhibition was much larger and voltage-dependent (Meir et al., 2000). It is therefore possible that phosphorylation of the  $Ca_v\beta$  subunit might mediate the loss of facilitation resulting from  $G_{\alpha_{qz5}}$  coexpression. Indeed, an-

other  $Ca_v\beta$  subunit,  $\beta 2a$ , is phosphorylated stoichiometrically by PKC (Puri et al., 1997). We will examine in a future study whether phosphorylation of  $Ca_v\beta$  subunits by PKC is responsible for the effects of  $G_{\alpha_{qz5}}$ .

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