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The α Subunit of G_q Contributes to Muscarinic Inhibition of the M-Type Potassium Current in Sympathetic Neurons

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Rat superior cervical ganglion (SCG) neurons express low-threshold noninactivating M-type potassium channels ($I_{K(M)}$), which can be inhibited by activation of M1 muscarinic receptors. This inhibition occurs via pertussis toxin-insensitive G-proteins belonging to the $G\alpha_q$ family (Caulfield et al., 1994). We have used DNA plasmids encoding antisense sequences against the 3′ untranslated regions of $G\alpha$ subunits (antisense plasmids) to investigate the specific G-protein subunits involved in muscarinic inhibition of $I_{K(M)}$. These antisense plasmids specifically reduced levels of the target G-protein 48 hr after intranuclear injection. In cells depleted of $G\alpha_q$, muscarinic inhibition of $I_{K(M)}$ was attenuated compared both with uninjected neurons and with neurons injected with an inappropriate $G\alpha_{oA}$ antisense plasmid. In contrast, depletion of $G\alpha_{11}$ protein did not alter $I_{K(M)}$ inhibition. To determine whether the α or $\beta\gamma$ subunits of the G-protein mediated this inhibition, we have overexpressed the C terminus of β adrenergic receptor kinase 1 (βARK1), which binds free $\beta\gamma$ subunits. βARK1 did not reduce muscarinic inhibition of $I_{K(M)}$ at a concentration of plasmid that can reduce $\beta\gamma$-mediated inhibition of calcium current (Delmas et al., 1998a). Also, expression of $\beta_1\gamma_2$ dimers did not alter the $I_{K(M)}$ density in SCG neurons. In contrast, $I_{K(M)}$ was virtually abolished in cells expressing GTPase-deficient, constitutively active forms of $G\alpha_q$ and $G\alpha_{11}$. These data suggest that $G\alpha_q$ is the principal mediator of muscarinic $I_{K(M)}$ inhibition in rat SCG neurons and that this more likely results from an effect of the α subunit than the $\beta\gamma$ subunits of the G_q heterotrimer.

Key words: M-current; G-protein; antisense; muscarinic receptor; superior cervical ganglion neuron; β adrenergic receptor kinase

The M-type potassium current ($I_{K(M)}$) is a noninactivating, voltage-gated potassium current found in various peripheral and central neurons, including rat superior cervical ganglion (SCG) neurons, and in some cell lines (for review, see Brown, 1988). It is activated in the subthreshold voltage range for action potentials and increases with membrane depolarization. Thus, cells remain clamped around rest, display spike adaptation, and have limited excitability. Inhibition of $I_{K(M)}$ results in depolarization with increased action potential discharge (Brown and Selyanko, 1985) and provides a switch between phasic and tonic firing properties (Wang and McKinnon, 1995). $I_{K(M)}$ in rat SCG neurons can be inhibited after activation of various receptors, including M_1 muscarinic receptors [M_1 mAChR (Marrion et al., 1989; Bernheim et al., 1992)] and bradykinin B_2 receptors (Jones et al., 1995), coupled through *Bordetella pertussis* toxin-insensitive GTP-binding proteins (G-proteins) (Brown et al., 1989; Caulfield et al., 1994; Jones et al., 1995).

Using antibodies raised against the C-terminal domain of different $G\alpha$ subunits, we have previously obtained evidence to suggest that the G-protein α subunits involved in M_1 mAChR-mediated inhibition of $I_{K(M)}$ in rat SCG neurons include $G\alpha_q$ or $G\alpha_{11}$ or both (Caulfield et al., 1994). However, the antibodies that were used could not distinguish between G_q and G_{11} because they have identical C-terminal sequences (Strathmann and Simon, 1990). Because the C terminus is thought to be a locus of G-protein GDP-bound α subunit/receptor and GTP-bound α subunit/phospholipase C-β1 (PLC-β1) interactions (Conklin and Bourne, 1993; Conklin et al., 1993; Arkinstall et al., 1995), $G\alpha_q$ and $G\alpha_{11}$ can couple to the same receptors (Aragay et al., 1992; Wu et al., 1992b; Nakamura et al., 1995; Dippel et al., 1996), and the cloned subunits stimulate the different PLC-β isoforms to a similar degree (Taylor et al., 1991; Hepler et al., 1993; Jhon et al., 1993). However, they are not invariably equivalent, because in rat portal vein myocytes, $G\alpha_q$ and $G\alpha_{11}$ elevate intracellular calcium levels after α_1-adrenoceptor activation by coupling to very different mechanisms (Macrez-Leprêtre et al., 1997).

In the present experiments, we have therefore tried to find out whether either or both of these two G-proteins (G_q and G_{11}) were involved in muscarinic inhibition of $I_{K(M)}$ in rat SCG neurons by using $G\alpha$ antisense-generating plasmids to deplete cells of specific subunits. We have also sought evidence to determine whether the α subunit or the $\beta\gamma$ dimer of the activated dissociated heterotrimer acted as the primary intermediary (Wickman and Clapham,

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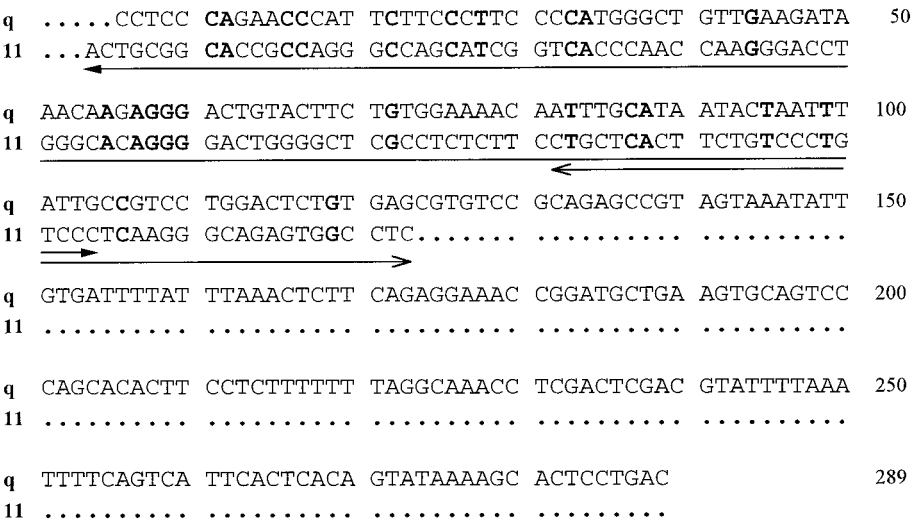

Figure 1. DNA Sequences of $G\alpha_q$ and $G\alpha_{11}$ 3′ untranslated regions. Sequences of rat $G\alpha_q$ and $G\alpha_{11}$ in the 3′ untranslated region immediately after the stop codon. Homology between the two proteins is very low in this region, with only 19% identity, although this rises to 31% when the two sequences are aligned for maximum homology. The *underlined areas* represent the sequences targeted by the $G\alpha_{11}$ antisense plasmids; the *closed arrowheads* correspond to clone 243–7 and the *open arrowheads* to clone C97–4.

1995; Clapham and Neer, 1997) by selectively overexpressing $\beta\gamma$ subunits or GTPase-deficient forms of the α subunits and by testing whether a $\beta\gamma$-sequestering agent [C-terminal peptide of β adrenergic receptor kinase 1 (βARK1)] modified the effect of mAChR stimulation.

Our results suggest that $G\alpha_q$, but not $G\alpha_{11}$, couples the M1 mAChR to $I_{K(M)}$ inhibition in SCG neurons and that α, rather than $\beta\gamma$, subunits are the mediators of this response.

MATERIALS AND METHODS

Cell culture. Sympathetic neurons were isolated from SCG of 15- to 19-d-old Sprague Dawley rats and cultured using standard procedures as described previously (Delmas et al., 1998a).

DNA plasmids. The constructs used in this study were made by PCR-cloning using standard molecular techniques (Abogadie et al., 1997). These were designed antisense to sequences in the 3′ untranslated (3′UT) regions of the rat target genes and subcloned into pCR3 or pCR3.1 (Invitrogen, San Diego, CA) unless stated otherwise. The cloned 3′UT sequences share no significant homology with any other rat G-protein α subunits. The nucleotide sequences reported in this paper have been submitted to the GenBank/EMBL Data Bank with accession numbers Y17161, Y17162, Y17163, and Y17164. The clones are as follows, in 5′ to 3′ orientation [nucleotide (nt); coding region (CR); numbers indicate position relative to stop or start codon]: $G\alpha_{oA}$ (clone 207–8) 3′UT nt 2–169: CTCTTGTCCTGTATAGCAACCTATTTGACTGCTTCATGGACTCTTTGCTGTTGATGTTGATCTCCTGGTAGCATGACCTTTGGCCTTTGTAAGACACACAGCCTTTCTGTACCAAGCCCCTGTCTAACCTACGACCCCAGAGTGACTGACGGCTGTGTATTTCTGTA; $G\alpha_{q/11\ common}$ (clone 107–6 in pBK-CMV, Stratagene, La Jolla, CA) CR nt 484–741: ATGACTTGGACCGTGTAGCCGACCCTTCCTATCTGCCTACACAACAAGATGTGCTTAGAGTTCGAGTCCCCACCACAGGGATCATTGAGTACCCCTTCGACTTACAGAGTGTCATCTTCAGAATGGTCGATGTAGGAGGCCAAAGGTCAG-AGAGAAGAAAATGGATACACTGCTTTGAAAACGTCACCTCGATCATGTTTCTGGTAGCGCTTAGCGAATACGATCAAGTTCT-TGTGGAGTCAGACAATGAGAACCGCA; $G\alpha_{11}$ antisense clones: 243–7, 3′UT nt 4–104; C97–4, 3′UT nt 82–123. $G\alpha_q$ antisense clones: C23–24, 3′UT nt 6–289; C6–6, 3′UT nt 6–129; C23-D7, 3′UT nt 193–289; C23–16, 3′UT nt 29–129. Targeted sequences are shown in Figure 1.

The constitutively active, GTPase-deficient form of hamster $G\alpha_q$ (Q209L) (Wu et al., 1992a) was subcloned into the pCMV5 vector, the GTPase-deficient $G\alpha_{11}$ (Q209L, also known as 11QL) (Wu et al., 1992a; from S. Offermanns) was provided in the pCIS vector, and the GTPase-deficient $G\alpha_{oA}$ (Q205L) (Wong et al., 1992; from B. R. Conklin) was provided in the pCDNA1 vector. Bovine β_1 and γ_2 subunits were subcloned into pCDNA3 (Invitrogen). The C-terminal Gly495 to Leu689 of human βARK1 (also called GRK2) (Koch et al., 1994; from C. Scorer and C. Harris) was supplied in the vector pCIN1 engineered with new *Not*I and *Eco*RI sites. All plasmids were propagated in either XL-1 blue or DH5α *Escherichia coli* and purified using maxiprep columns (Qiagen, Hilden, Germany). All clones were verified by sequencing. RNA synthesis was driven by a strong viral promoter (cytomegalovirus) to ensure sustained high intracellular levels of transcripts after delivery of plasmids.

In situ *hybridization. In situ* hybridization was performed on 12 μm cryostat sections of 17-d-old rat SCGs using digoxigenin-labeled riboprobes, essentially as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993). Sense and antisense cRNAs were transcribed from the same clones used in the electrophysiological experiments using SP6 and T7 polymerase according to standard protocols.

Microinjection. DNA plasmids were diluted to 400 μg/ml in calcium- and glucose-free Krebs' solution (290 mOsm/l, pH 7.3) containing 0.5% FITC-dextran and pressure-injected into the nucleus of SCG neurons 2 d in culture, either as described previously (Abogadie et al., 1997) or with a microinjector (Eppendorf, Hamburg, Germany). Cells were maintained in culture for an additional 2 d, and a survival rate of 75–85% was obtained.

Electrophysiology. M-currents were measured from SCG neurons cultured for 4 d, using the amphotericin-B perforated-patch technique (Horn and Marty, 1988; Rae et al., 1991). Patch electrodes (2–4 MΩ) were filled by dipping the tip for 40 sec into a filtered internal solution containing (in mM): potassium acetate 80, KCl 30, HEPES 40, $MgCl_2$ 3 (adjusted to pH 7.3–7.4 with KOH and to 280 mOsm/l with potassium acetate). The pipette was then back-filled with the above solution containing 0.1 mg/ml amphotericin-B. High-resistance seals (>2 GΩ) were initially achieved, and after amphotericin-B permeabilization, access resistances were <25 MΩ. SCG neurons were perfused at 5–10 ml/min at 32°C with an external solution consisting of (in mM): NaCl 120, KCl 3, HEPES 5, $NaHCO_3$ 23, glucose 11, $MgCl_2$ 1.2, $CaCl_2$ 2.5, tetrodotoxin (TTX) 0.0005, pH 7.4. Cells were voltage-clamped at approximately −25 mV using either an Axopatch 200A amplifier (data sampling rate 4–10 kHz, filter 1 kHz) or a switching amplifier (Axoclamp-2A, switching frequencies 3–5 kHz, filter 0.1 kHz), both from Axon instruments (Foster City, CA). $I_{K(M)}$ was measured as a slowly developing inward deactivation relaxation after a 1 sec jump to a command potential of approximately −55 mV (Caulfield et. al., 1994). Inhibition was measured as the fractional reduction in the amplitude of the $I_{K(M)}$ deactivation relaxation in response to cumulative increases in concentrations of oxotremorine methiodide (Oxo-M) (Research Biochemicals International, Natick, MA) (see Fig. 4). Steady-state current–voltage relationships were obtained by applying slow (3.3 mV/sec) voltage ramps from −20 mV to −100 mV. For experiments with *Bordetella pertussis* toxin (PTX) (Speywood, Maidenhead, Berkshire, UK), SCG neurons were incubated with 1 μg/ml PTX in the culture medium for at least 24 hr before recording.

Data were collected and analyzed using PClamp6 software (Axon Instruments) and expressed as mean ± SEM. An estimate of the mean log IC_{50} for each antisense plasmid treatment was obtained by fitting the data from each individual cell with a best-fit dose–response curve and determining the log IC_{50} for each cell. $I_{K(M)}$ deactivation relaxations were best-fit by a double exponential with fast (τ1) and slow (τ2) components. Statistics used the two-way ANOVA comparing plasmid treatments across four agonist concentrations for all antisense samples (in-

cluding uninjected groups). If a significant effect of plasmid treatment was found overall, further analysis was performed using the two-way ANOVA to determine which treatments contributed to this significance. The constitutively active $G\alpha_{oA^*}$, $G\alpha_{q^*}$, $G\alpha_{11^*}$, and $\beta_1\gamma_2$ expression data were analyzed with one-way ANOVA, as was the log IC_{50} data, and if an overall significant effect of plasmid treatment was found, this was followed by Bonferroni's multiple comparison test. p values < 0.05 were considered significant.

Immunocytochemistry. SCG cells, cultured and injected as described above, were fixed in acetone and stained for $G\alpha_{oA+B}$, $G\alpha_q$, $G\alpha_{11}$, and C terminus of βARK1 using selective antibodies and the alkaline phosphatase substrate 5-bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride (BCIP/NBT) (Dako, Carpinteria, CA), as described by Abogadie et al., (1997). The polyclonal antibodies anti-$G\alpha_{oA+B}$ (sc-387), anti-$G\alpha_{11}$ (sc-394), and anti-βARK1 C terminus (sc-562) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the anti-Gβ antibody (3B-200) was from Gramsch Laboratories (Schwabhausen, Germany). The specific polyclonal antibody anti-$G\alpha_q$ (IQB2) was raised against a synthetic peptide fragment of $G\alpha_q$ (Milligan et al., 1993). Specificity of the antibodies was determined by competing out the staining by preabsorbing the antibody with the relevant immunogenic peptide. All dishes of SCG neurons recorded in the electrophysiology experiments were subsequently fixed and stained. The BCIP/NBT purple/blue product was too dark to quantitate photometrically, so we assessed whether there was an overall qualitative reduction in staining by comparing each injected cell with its nearest uninjected neighbor and determining (by eye) whether the level of staining was equal to or less than that of the uninjected cell. Using this method we have therefore estimated the proportion of cells with a visible reduction in staining (regardless of the magnitude of this reduction) 48 hr after injection of the antisense plasmid.

RESULTS

$G\alpha_q$ and $G\alpha_{11}$ expression in SCG neurons

Both *in situ* hybridization and RT-PCR clearly showed the presence of $G\alpha_q$ and $G\alpha_{11}$ mRNAs in rat SCG tissue where they were expressed mainly in neurons (Fig. 2). The specificity of the hybridization probes was confirmed when no signal was seen after competition with unlabeled probes (data not shown) or after use of sense, rather than antisense, probes (Fig. 2). Staining with specific antibodies against $G\alpha_q$ and $G\alpha_{11}$ demonstrated the presence of $G\alpha_q$ and $G\alpha_{11}$ protein in most cultured SCG neurons (see below). We have constructed plasmids encoding for RNA antisense (antisense plasmids) directed against the 3′ untranslated region of these G-proteins to specifically deplete cells of each of the α-subunits. $G\alpha_q$ and $G\alpha_{11}$ share 81% homology in coding region sequence (based on mouse sequence), and this drops to a maximum of 31% (when rat sequences are aligned for maximum homology) in the first 200 bases of the 3′ untranslated region in rat (Fig. 1).

Direct intranuclear injection of SCG neurons with various antisense plasmids ($G\alpha_q$, $G\alpha_{11}$, and $G\alpha_{oA}$) resulted in a marked reduction in the respective Gα subunit staining 48–72 hr later (Fig. 3). This protein depletion was specific, with $G\alpha_{oA}$ antisense not altering $G\alpha_q$ or $G\alpha_{11}$ staining, $G\alpha_{q/11\ common}$ antisense not touching $G\alpha_{oA+B}$ staining, $G\alpha_q$ not altering $G\alpha_{oA+B}$ or $G\alpha_{11}$ staining, and $G\alpha_{11}$ antisense leaving $G\alpha_{oA+B}$ and $G\alpha_q$ staining intact. The two $G\alpha_{11}$ antisense plasmids, however, were not equally effective. Thus, clone C97–4 reduced visible $G\alpha_{11}$ protein staining in 9 of 19 cells (47%; $n = 7$ dishes of cells) (see Materials and Methods) (Fig. 3*C*), whereas 243–7 reduced $G\alpha_{11}$ staining in only 18 of 63 cells (29%; $n = 17$ dishes). Similarly, the specific $G\alpha_q$ antisense plasmids were not all effective. C6–6 and C23–24 reduced staining in 23 of 71 cells (32%; $n = 8$ dishes) and 8 of 32 cells (25%; $n = 6$ dishes), respectively (Fig. 3*B*), whereas C23-D7 and C23–16 were more effective, reducing staining in 31 of 65 cells (48%; $n = 10$ dishes) and 55 of 109 cells (50%; $n = 21$ dishes), respectively (Fig. 3*B*). The antisense plasmids were maximally effective in reducing Gα subunit staining 2 d after injection, and their effects on $I_{K(M)}$ modulation were therefore assessed 2 d after injection.

Effect of antisense plasmids on $I_{K(M)}$ modulation by a muscarinic agonist

Injection of DNA plasmids encoding antisense to $G\alpha_{oA}$ slightly reduced inhibition of $I_{K(M)}$ by the muscarinic agonist Oxo-M. Thus, the inhibition of $I_{K(M)}$ by 300 nM Oxo-M was 24.6 ± 5.0% ($n = 6$) in $G\alpha_{oA}$ antisense-treated cells compared with 34.2 ± 2.6% ($n = 7$) in uninjected cells ($p = 0.007$ across all Oxo-M concentrations) (see Fig. 5*B*). To investigate further this effect of $G\alpha_{oA}$ antisense, we pretreated several dishes of SCG neurons with 1 μg/ml PTX, which ADP-ribosylates and inactivates members of the $G\alpha_{o/i}$ G-protein family. There was no significant difference in Oxo-M inhibition of $I_{K(M)}$ between the treated and untreated cells (see Fig. 5*D*), confirming previous findings with other mAChR agonists (Brown et al., 1989). A comparable treatment strongly attenuated the G_o-mediated inhibition of the Ca^{2+} current by noradrenaline (Caulfield et al., 1994) and Oxo-M (Delmas et al., 1998b). Furthermore, overexpression of a constitutively active, GTPase-deficient form of $G\alpha_{oA}$ (Wong et al., 1992) did not alter $I_{K(M)}$ density (see Fig. 8 and below). It seems unlikely, therefore, that the $G\alpha_{oA}$ antisense-induced reduction in $I_{K(M)}$ inhibition results directly from the loss of $G\alpha_{oA}$ or that $G\alpha_{oA}$ participates in $I_{K(M)}$ inhibition, a conclusion supported by previous studies using specific antibodies (Caulfield et al., 1994). This reduction is also unlikely to be caused by the plasmid injection per se, because cells injected with antisense constructs that were ineffective in reducing protein had no effect on the Oxo-M dose–response curves (see Fig. 5*B,C* and below). Hence, we do not yet understand why the $G\alpha_{oA}$ antisense plasmid reduced $I_{K(M)}$ inhibition. Nevertheless, because the most suitable control group for comparison with the $G\alpha_q$ and $G\alpha_{11}$ antisense plasmids is the expression of an inappropriate antisense, we have taken the effect of the $G\alpha_{oA}$ antisense as our baseline for assessing the effect of the $G\alpha_q$ and $G\alpha_{11}$ antisense plasmids, because at the very least this would mitigate against any "nonspecific" effects of antisense plasmid injection. Thus, all p values quoted are compared against $G\alpha_{oA}$ antisense-expressing neurons unless stated otherwise (in practice, the same outcome of the experiments below would be obtained if the comparison were with uninjected cells).

Injection of SCG neurons with the $G\alpha_{q/11\ common}$ antisense plasmid significantly reduced Oxo-M inhibition of $I_{K(M)}$ when compared with $G\alpha_{oA}$ antisense-expressing cells ($G\alpha_{oA}$ antisense: 24.6 ± 5.0% inhibition with 300 nM Oxo-M, $n = 6$; $G\alpha_{q/11}$ antisense: 13.9 ± 2.2%, $n = 6$; $p = 0.005$ across all Oxo-M concentrations) (see Fig. 5*B*). This confirms previous observations, using functionally inactivating antibodies, that either $G\alpha_q$ or $G\alpha_{11}$ or both mediate muscarinic inhibition of $I_{K(M)}$ (Caulfield et al., 1994). To determine which (or whether both) of these G-protein α subunits is responsible for mediating this response, cells were injected with antisense plasmids that specifically reduced $G\alpha_q$ and $G\alpha_{11}$ levels (see above). Four plasmids encoding different $G\alpha_q$ antisense sequences were investigated (see Fig. 5*A*). Of these, two significantly reduced muscarinic inhibition of $I_{K(M)}$ (C23-D7 and C23–16) (percentage inhibition with 300 nM Oxo-M: C23-D7: 15.5 ± 4.7%, $n = 6$, $p = 0.004$ compared with $G\alpha_{oA}$ dose–response curve; C23–16: 15.7 ± 3.7%, $n = 9$, $p = 0.001$) (Figs. 4, 5*C*). This is in agreement with the immunocytochemical

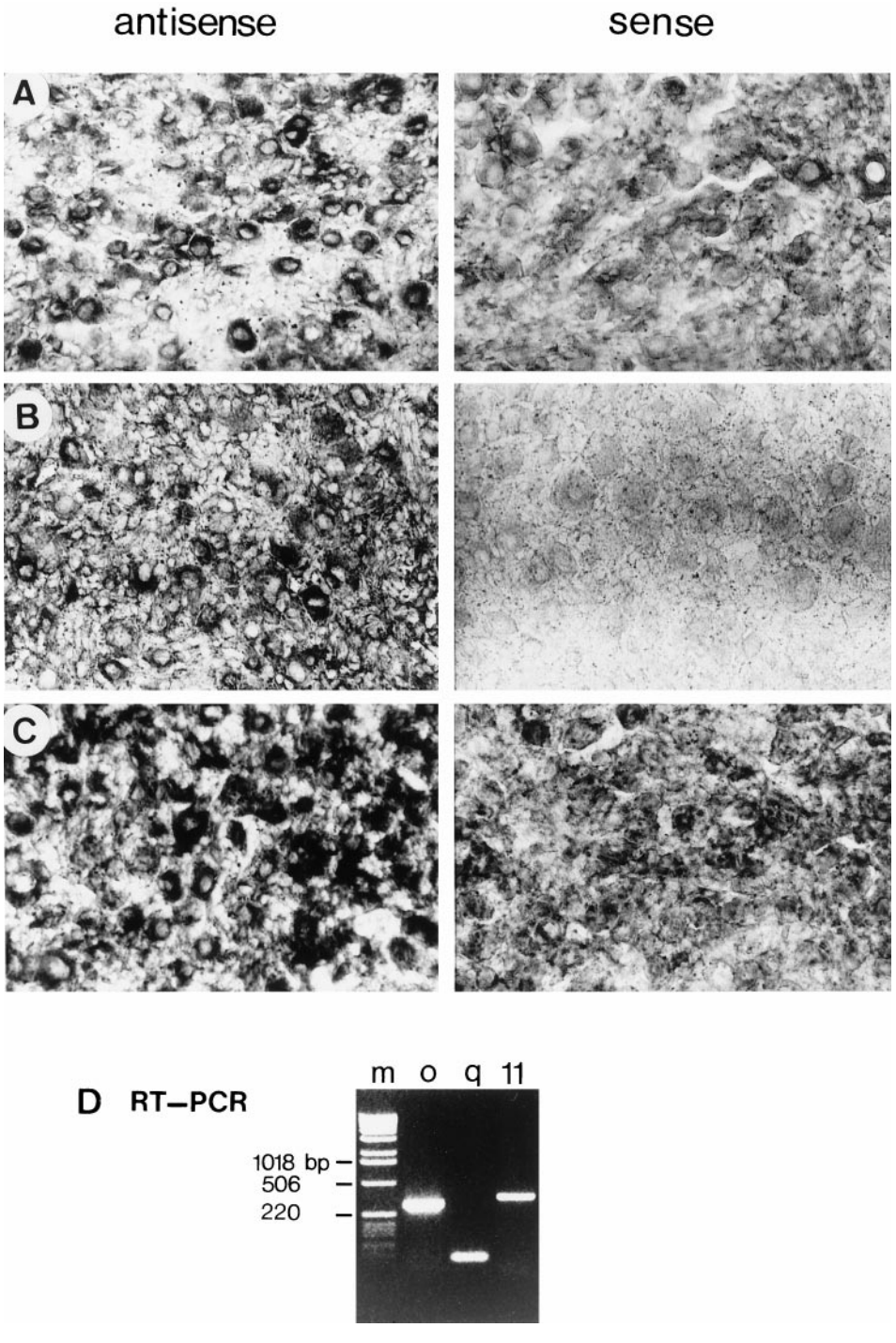

Figure 2. *In situ* hybridization and RT-PCR demonstrate the presence of $G\alpha_q$ and $G\alpha_{11}$ mRNA in rat SCG. *In situ* hybridization (ISH) and RT-PCR demonstrate the presence of $G\alpha_q$ and $G\alpha_{11}$ in rat SCG. ISH of $G\alpha_q$ (*B*) and $G\alpha_{11}$ (*C*) shows neuronal staining. $G\alpha_{oA}$ ISH (*A*) was used as a positive control. All probes used were against the 3′ untranslated region (3′ UTR) of the gene. The *black arrows* indicate representative individual SCG neurons expressing the relevant mRNA. *D*, RT-PCR using rat SCG DNA as a template. *m*, Marker lane; *o*, $G\alpha_o$ with primers 266s/849a; *q*, $G\alpha_q$ with primers $G\alpha_q$ u6s/u111a, where "u" denotes sequence in the 3′ UTR; *11*, $G\alpha_{11}$ with primers $G\alpha_{11}$ 488s/u103a; *bp*, base pair.

data where the clones C23-D7 and C23–16 selectively reduced immunocytochemical staining of $G\alpha_q$. The other two clones, C6–6 and C23–24, however, were less effective at reducing either $G\alpha_q$ staining or muscarinic inhibition of $I_{K(M)}$ (percentage inhibition of $I_{K(M)}$ with 300 nM Oxo-M: C6–6: 24.3 ± 5.0%, $n = 8$; C23–24: 24.3 ± 3.0%, $n = 5$) (Fig. 5*C*). The attenuation of $I_{K(M)}$ inhibition by the $G\alpha_q$ antisense plasmids C23-D7 and C23–16 was reflected in an increase in the log IC_{50} for these groups. Although the log IC_{50} values for neurons injected with C6–6 (−6.14 ± 0.09; $n = 8$) and C23–24 (−6.07 ± 0.11; $n = 5$) were close to that for uninjected neurons (−6.31 ± 0.06; $n = 6$), those for SCG neurons injected with the $G\alpha_q$ antisense plasmids C23-D7 (−5.64 ± 0.23; $n = 6$) and C23–16 (−5.16 ± 0.37; $n = 7$; $p < 0.05$ compared with $G\alpha_{11}$ antisense-expressing neurons) were greater (Fig. 6). $G\alpha_q$ depletion did not significantly change the maximum response or Hill slope. In contrast with the $G\alpha_q$ antisense plasmids, $I_{K(M)}$ inhibition in cells depleted of $G\alpha_{11}$ with C97–4 antisense plasmid (29.7 ± 4.1% inhibition at 300 nM Oxo-M; log IC_{50} = −6.13 ± 0.09; $n = 9$) was no different from that seen in $G\alpha_{oA}$ antisense-expressing neurons (Figs. 4, 5*B*, 6). In agreement with the time course of $G\alpha$ subunit depletion, the reduction of Oxo-M inhibition was maximal at 48 hr for the concentration of the plasmid injected (400 μg/ml), because no further reduction was seen 72 hr after injection [e.g., at 72 hr, 300 nM Oxo-M produced 17.4 ±

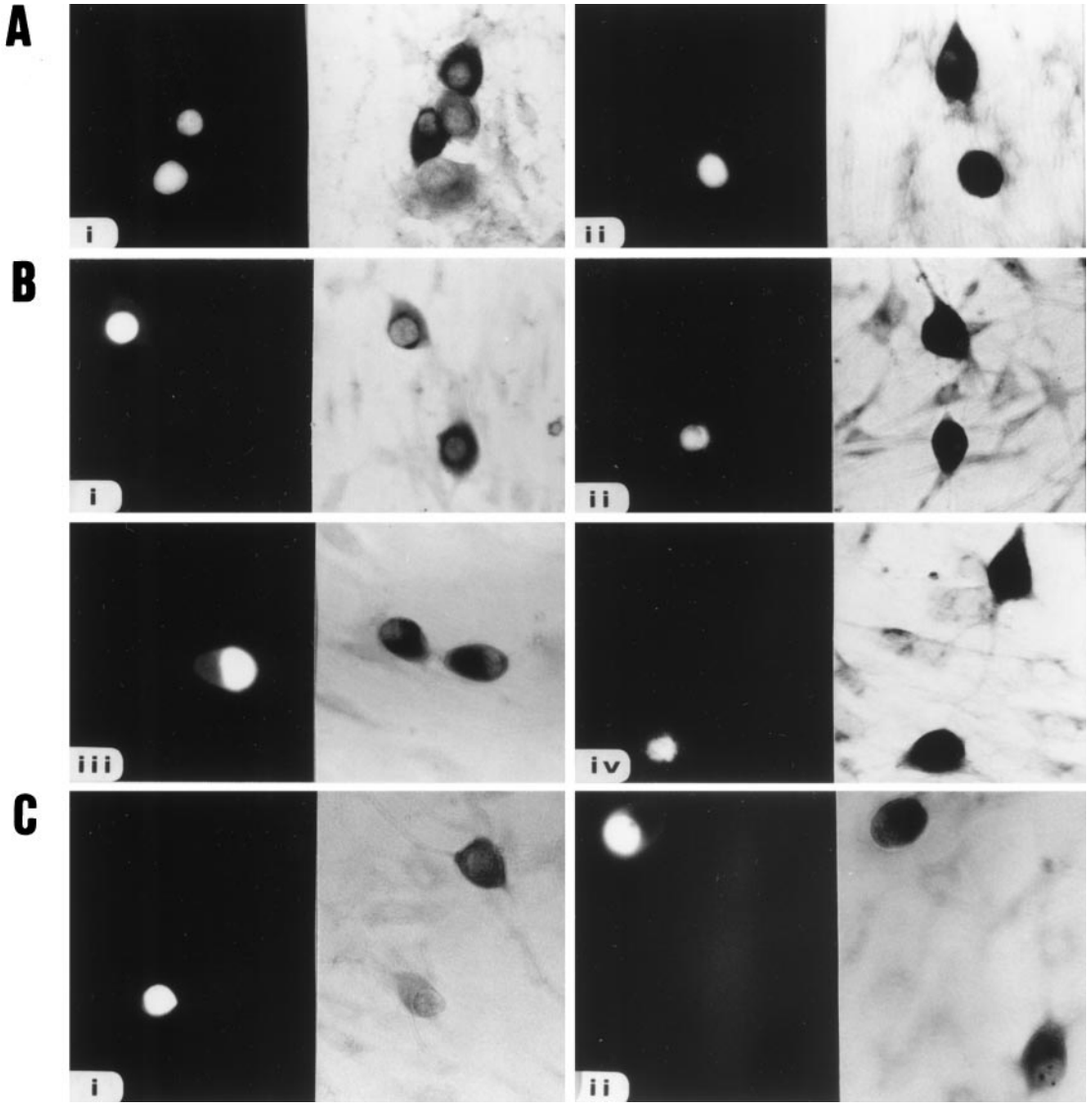

Figure 3. Reduction of G-protein α subunit staining in cells expressing antisense. Complementary fluorescence and $G\alpha$ immunostaining photographs of cells intranuclearly injected with antisense plasmids and a fluorescent marker. *A*, Cells immunostained with $G\alpha_{oA+B}$ antibody and injected with (*i*) $G\alpha_{oA}$ antisense plasmid and (*ii*) $G\alpha_q$ antisense plasmid (clone C23-D7). *B*, Cells immunostained with $G\alpha_q$ antibody and neurons injected with (*i*) $G\alpha_q$ antisense plasmid (C23-D7), (*ii*) $G\alpha_{oA}$ antisense plasmid, (*iii*) $G\alpha_q$ antisense plasmid (C23–24), and (*iv*) $G\alpha_{11}$ antisense plasmid (C97–4). *C*, Cells immunostained with $G\alpha_{11}$ antibody and neurons injected with (*i*) $G\alpha_{11}$ antisense plasmid (C97–4) and (*ii*) $G\alpha_q$ antisense plasmid (C23-D7).

2.7% ($n = 9$) inhibition of $I_{K(M)}$ in $G\alpha_q$ antisense (C23–16)-expressing cells)]. The resting membrane potential was not altered in neurons injected with the $G\alpha_q$ or $G\alpha_{11}$ antisenses compared with $G\alpha_{oA}$ antisense constructs (e.g., $G\alpha_{oA}$ antisense: -63.5 ± 2.6 mV, $n = 6$; $G\alpha_q$ antisense, C23-D7: -63.8 ± 1.1 mV, $n = 5$; $G\alpha_{11}$ antisense, C97–4: -62.0 ± 4.0 mV, $n = 4$).

Expression of the C-terminal βARK1 peptide in SCG neurons

The above results suggest that $G\alpha_q$ is primarily responsible for M1 mAChR-induced inhibition of $I_{K(M)}$, but they do not indicate which subunit(s) of the heterotrimer mediates the inhibition. To determine the role of endogenous $G\alpha_q$-linked $\beta\gamma$ dimers, we overexpressed the C-terminal domain of βARK1, which has been shown to sequester free $\beta\gamma$ subunits (Koch et al., 1994). Expression of the peptide was routinely detected 24–48 hr after injection as a strong increase in βARK1 peptide immunoreactivity. Injection of the βARK1 construct at 200 μg/ml, a concentration that has been found to be effective at attenuating noradrenergic inhibition of the calcium current (Delmas et al., 1998a), a presumed $\beta\gamma$-mediated pathway (Herlitze et al., 1996; Ikeda, 1996), did not alter inhibition of $I_{K(M)}$ (300 nM Oxo-M produced $21.8 \pm 3.2\%$ inhibition in βARK1-expressing cells; $n = 8$) (Fig. 7). Increasing the plasmid concentration to 400 μg/ml, however, resulted in a reduction of M1 mAChR inhibition of $I_{K(M)}$ (300 nM Oxo-M resulted in $17.7 \pm 2.4\%$ inhibition, $n = 7$, $p = 0.0002$, compared with $G\alpha_{oA}$ antisense plasmid cells, across all concentrations of Oxo-M) (Fig. 7). This effect was not a result of a use-dependent sequestration of $\beta\gamma$ subunits by βARK1 peptide, because repetitive application of 1 μM Oxo-M did not result in an accumulated loss of inhibition in neurons injected with 400 μg/ml βARK1-encoding plasmid (first application, $40.5 \pm 4.0\%$ inhibition; fourth application, $37.7 \pm 4.1\%$; $n = 3$). Furthermore, neither $I_{K(M)}$ current density ($G\alpha_{oA}$ anti-

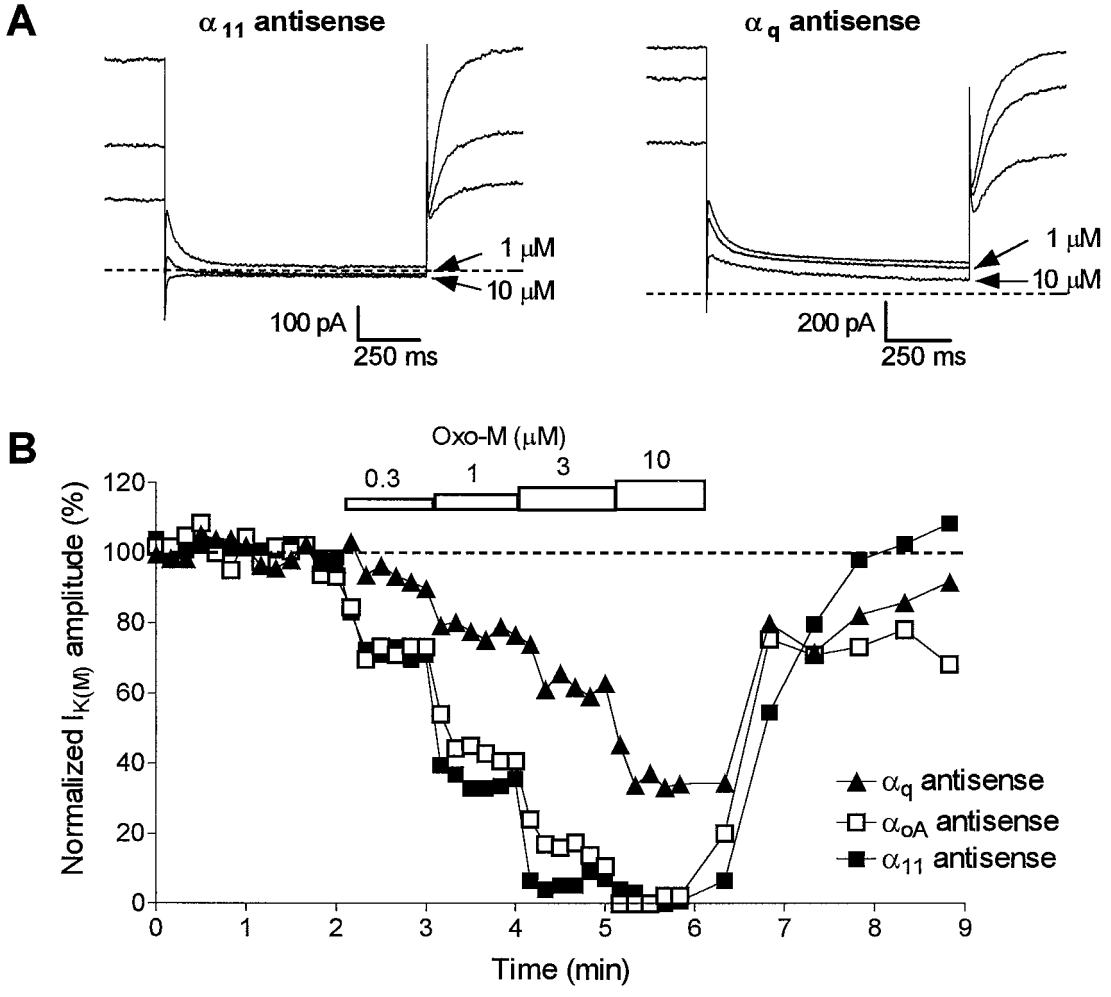

Figure 4. Time course of cumulative Oxo-M application; effect on $I_{K(M)}$ amplitude. *A*, $I_{K(M)}$ deactivation relaxation elicited by a −30 mV step for 1 sec from a holding potential of approximately −25 mV. Waveforms (average of 3 traces) are from cells injected with $G\alpha_{11}$ (C97–4) or $G\alpha_q$ (C23–16) antisense plasmids and whose time courses are shown in *B*. $I_{K(M)}$ relaxations are shown in the absence and presence of 1 μM and 10 μM Oxo-M. *Dotted lines* represent 0 pA. *B*, Time course of normalized $I_{K(M)}$ amplitude during application of increasing concentrations of Oxo-M, as indicated, for neurons injected with $G\alpha_{oA}$, $G\alpha_q$, and $G\alpha_{11}$ antisense plasmids. $I_{K(M)}$ was recorded every 10 sec, and each Oxo-M concentration was applied for 1 min.

sense: 2.8 ± 0.4 pA/pF, $n = 7$; 400 μg/ml βARK1: 4.7 ± 1.1 pA/pF, $n = 7$) nor $I_{K(M)}$ deactivation relaxation ($G\alpha_{oA}$ antisense: τ_1 40.3 ± 2.3 msec, τ_2 263 ± 21 msec, $n = 9$; 400 μg/ml βARK1: τ_1 37.2 ± 2.2 msec, τ_2 248 ± 30 msec, $n = 10$) was significantly altered by the βARK1-encoding plasmid.

Expression of GTPase-deficient forms of $G\alpha_q$ and $G\alpha_{11}$ subunits, but not $\beta_1\gamma_2$ dimers, inhibits $I_{K(M)}$

The above experiments with βARK1 peptide expression suggest that α, rather than βγ, subunits mediate M1 mAChR inhibition of $I_{K(M)}$. To test this further, we overexpressed GTPase-deficient, constitutively active forms of $G\alpha_{oA}$ ($G\alpha_{oA^*}$, Q205L), $G\alpha_q$ ($G\alpha_{q^*}$, Q209L), $G\alpha_{11}$ ($G\alpha_{11^*}$, Q209L), and $\beta_1\gamma_2$ dimers. Overexpression of $G\alpha_{q^*}$ and $G\alpha_{11^*}$ resulted in a dramatic decrease in $I_{K(M)}$ current density 24–48 hr after injection, compared with cells injected with $G\alpha_{oA}$ antisense plasmid or $G\alpha_{oA^*}$ (Fig. 8*A,C*) ($G\alpha_{oA}$ antisense: 2.8 ± 0.4 pA/pF, $n = 7$; $G\alpha_{oA^*}$: 4.0 ± 0.6 pA/pF, $n = 6$; $G\alpha_{q^*}$: 0.2 ± 0.02 pA/pF, $n = 13$, $p < 0.001$, compared with either $G\alpha_{oA}$ antisense or $G\alpha_{oA^*}$; $G\alpha_{11^*}$: 0.1 ± 0.02 pA/pF, $n = 8$, $p < 0.001$, compared with either $G\alpha_{oA}$ antisense or $G\alpha_{oA^*}$). This loss of $I_{K(M)}$ was also clear in the steady-state current–voltage relationships by the absence of outward rectification positive to −60 mV in $G\alpha_{q^*}$- and $G\alpha_{11^*}$-expressing cells (Fig. 8*B*). Consistent with the suppression of $I_{K(M)}$, the resting membrane potential of these cells was more depolarized than in cells injected with $G\alpha_{oA}$ antisense and $G\alpha_{oA^*}$ plasmids ($G\alpha_{oA}$ antisense: −63.5 ± 2.6 mV, $n = 6$; $G\alpha_{oA^*}$: −62.7 ± 0.7 mV, $n = 6$; $G\alpha_{q^*}$: −48.4 ± 1.7 mV, $n = 13$, $p < 0.001$, compared with either $G\alpha_{oA}$ antisense or $G\alpha_{oA^*}$; $G\alpha_{11^*}$: −50.3 ± 2.7 mV, $n = 8$, $p < 0.01$, compared with $G\alpha_{oA}$ antisense or $G\alpha_{oA^*}$). In contrast, overexpression of free βγ subunits, by coexpressing β_1 and γ_2 subunits, had no significant effect on either $I_{K(M)}$ current density (2.03 ± 0.4 pA/pF, $n = 6$; $p > 0.05$ compared with $G\alpha_{oA}$ antisense) (Fig. 8*C*) or resting membrane potential (−59.2 ± 1.8, $n = 6$; $p > 0.05$, compared with $G\alpha_{oA}$ antisense).

DISCUSSION

Our data clearly demonstrate that direct intranuclear injection of antisense-generating plasmids is an effective method for reducing levels of G-protein subunits in neurons (Fig. 3). These antisense sequences, designed against the 3′ UTR for increased specificity, are thought to bind to their target regions and destabilize the whole mRNA (Phillips and Gyurko, 1997), resulting in a reduced level of expression of the target protein. It is interesting to note

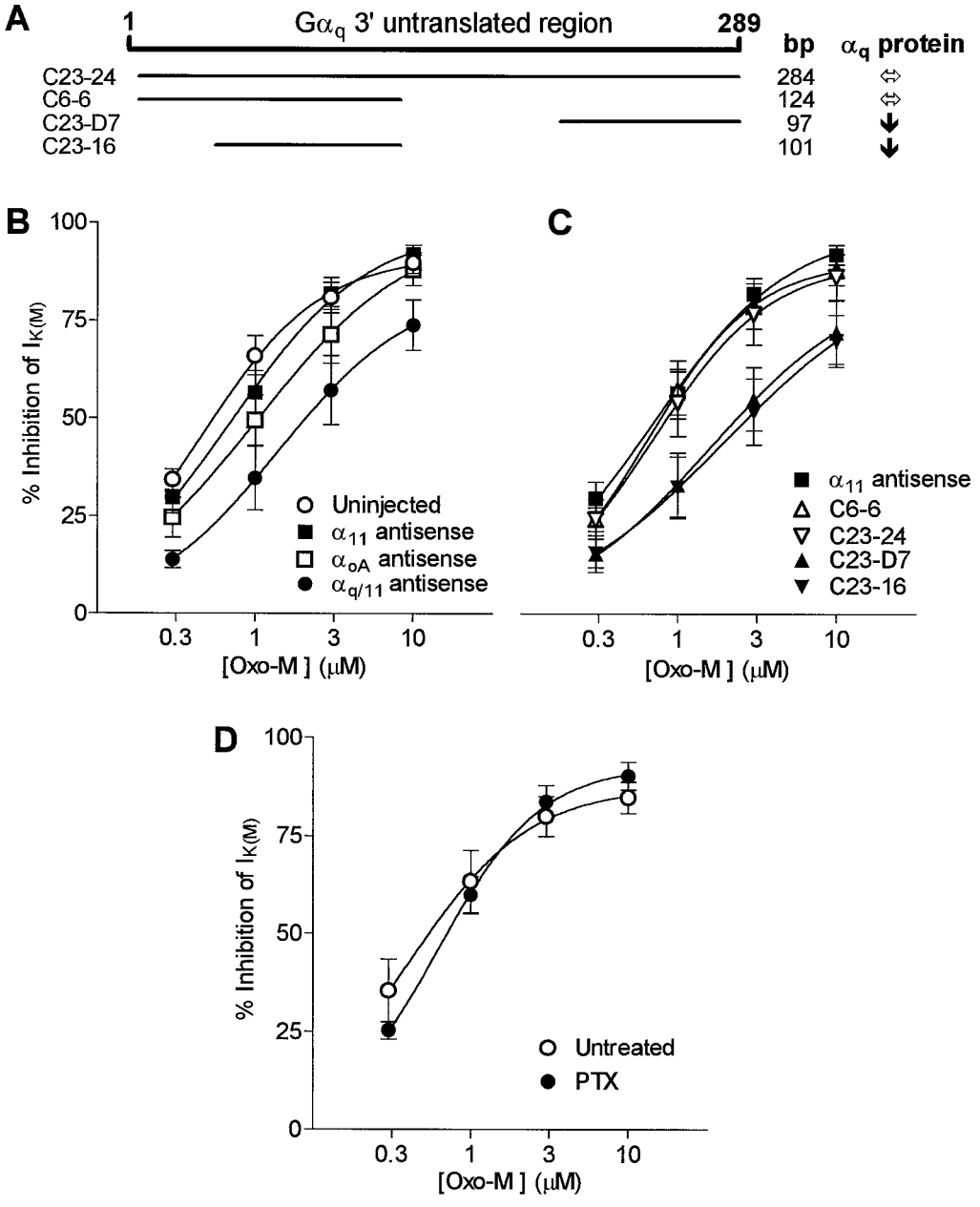

Figure 5. Dose–response curves for Oxo-M inhibition of $I_{K(M)}$ in antisense plasmid-injected SCG neurons. *A*, Schematic diagram demonstrating length [in base pairs (bp)] and relative positions of the four antisense sequences targeted at the 3′ untranslated region of rat $G\alpha_q$. Only C23-D7 and C23–16 consistently reduced $G\alpha_q$ protein levels in immunocytochemical staining with a $G\alpha_q$ antibody. *B*, Dose–response curves (mean ± SEM, plus best-fit curve) for uninjected neurons compared with $G\alpha_{oA}$ antisense, $G\alpha_{11}$ antisense, and $G\alpha_{q/11}$ antisense plasmid-injected cells. The dose–response curve for $G\alpha_{oA}$ ($n = 6$) antisense is significantly different from the uninjected dose–response curve ($n = 6$; $p = 0.007$) but not the $G\alpha_{11}$ antisense plasmid curve ($n = 9$). The dose–response curve for cells injected with the $G\alpha_{q/11\ common}$ antisense plasmid ($n = 6$) is significantly different from those of cells injected with $G\alpha_{oA}$ and $G\alpha_{11}$ antisense plasmids ($p = 0.005$ and $p < 0.0001$, respectively). *C*, Oxo-M dose–response curves for neurons injected with $G\alpha_{11}$ antisense plasmid and four different antisense plasmids against $G\alpha_q$. The dose–response curves for C6–6 ($n = 8$) and C23–24 ($n = 5$) are not significantly different from the dose–response curves for $G\alpha_{oA}$ antisense-expressing (*B*) or $G\alpha_{11}$ antisense-expressing neurons. C23-D7 ($n = 6$) and C23–16 ($n = 9$) dose–response curves are both significantly different from $G\alpha_{oA}$ antisense-expressing neurons ($p = 0.004$ and $p = 0.001$, respectively) and from $G\alpha_{11}$ antisense-expressing neurons ($p < 0.0001$ and $p < 0.0001$, respectively). *D*, Oxo-M inhibition of $I_{K(M)}$ is not altered by pretreatment with 1 μg/ml PTX ($n = 6$; $n = 4$ for untreated neurons).

that not all $G\alpha$ 3′ UTR antisense sequences were effective in reducing protein expression. Of four $G\alpha_q$ antisense sequences tested, only two were effective (C23-D7 and C23–16). Similarly, of two $G\alpha_{11}$ antisense sequences tested, only one (C97–4) consistently reduced $G\alpha_{11}$ protein levels. This difference in effectiveness of the antisense sequences could perhaps arise from some unknown secondary structure in the target mRNA transcript (Phillips and Gyurko, 1997). Such structures may determine how accessible the target region is for binding by antisense transcripts. The two less effective $G\alpha_q$ antisenses include a short region between nt 6 and 28 in the 3′ UTR not covered by the other antisense sequences (Fig. 5). It is therefore tempting to speculate

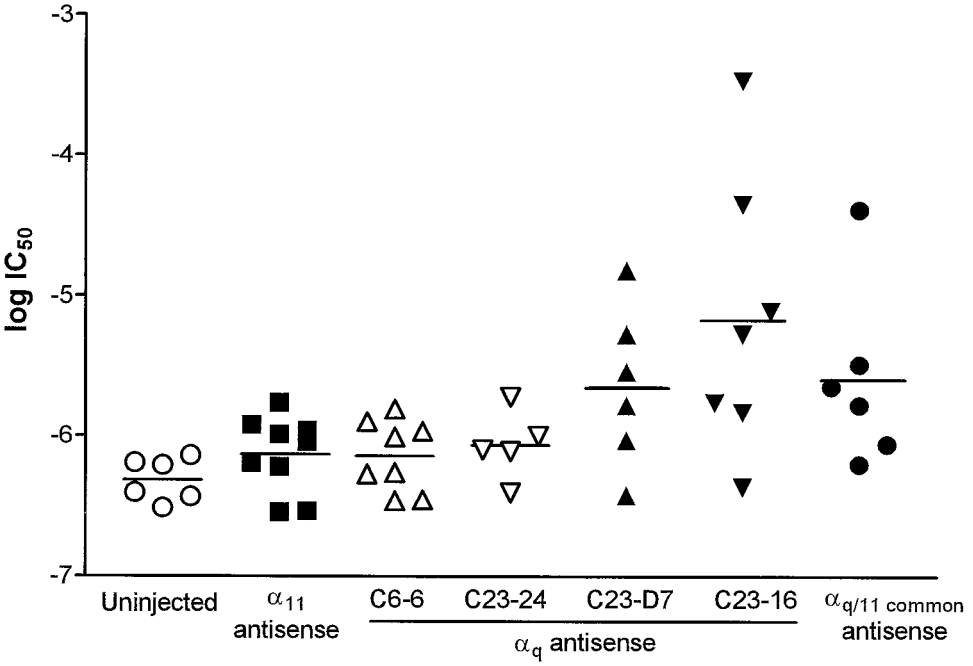

Figure 6. $G\alpha_q$ antisense plasmids increase the log IC_{50} for Oxo-M inhibition of $I_{K(M)}$. Scatter-plot is shown of the log IC_{50} for each neuron included in the mean dose–response curves in Figure 5. Log IC_{50} was calculated from the best-fit curve for Oxo-M inhibition of $I_{K(M)}$ for every neuron recorded with the injected antisense sequences indicated. *Horizontal lines* represent the mean of each group. The $G\alpha_q$ antisense plasmid C23–16 significantly increased the log IC_{50} compared with uninjected neurons ($p < 0.01$), $G\alpha_{11}$ antisense plasmid injected cells ($p < 0.05$), and cells injected with the $G\alpha_q$ antisense plasmid C6–6 ($p < 0.05$).

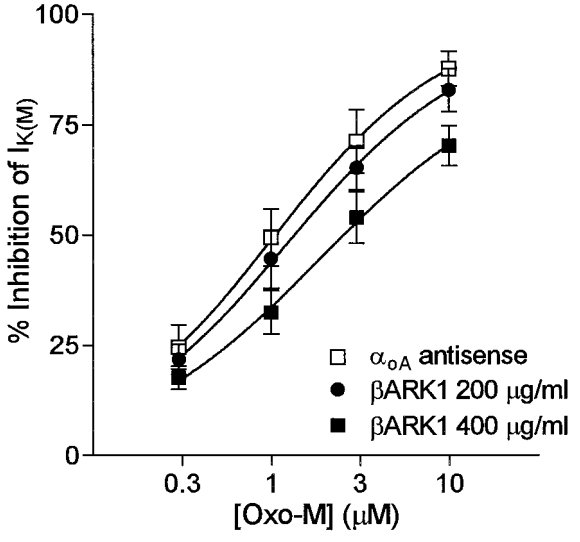

Figure 7. βARK1-injected cells show some attenuation of $I_{K(M)}$ inhibition by Oxo-M. Dose–response curves for Oxo-M inhibition of $I_{K(M)}$ in cells injected with either $G\alpha_{oA}$ antisense plasmid ($n = 6$) or C-terminal βARK1 plasmid at 200 μg/ml ($n = 8$) or 400 μg/ml ($n = 7$) are shown. Only the βARK1 400 μg/ml dose–response curve is significantly different from the $G\alpha_{oA}$ antisense dose–response curve ($p = 0.0002$).

that this particular region or sequence might not be amenable to antisense action.

Using these specific antisense plasmids, we have shown that depletion of $G\alpha_q$, but not $G\alpha_{11}$, subunits significantly reduced muscarinic inhibition of $I_{K(M)}$ (Figs. 4, 5). Indeed, although both *in situ* hybridization and immunocytochemical experiments clearly demonstrated the presence of $G\alpha_{11}$ in SCG neurons (Figs. 2, 3), muscarinic inhibition of $I_{K(M)}$ was not altered in cells specifically depleted of $G\alpha_{11}$ by the antisense plasmid (Figs. 3–6). This suggests that $G\alpha_q$ rather than $G\alpha_{11}$ preferentially mediates M1 mAChR inhibition of $I_{K(M)}$. This idea is supported by the finding that the common $G\alpha_{q/11}$ antisense plasmid produced no greater reduction in $I_{K(M)}$ inhibition than the specific $G\alpha_q$ antisense plasmids. Although $G\alpha_q$ antisense plasmids clearly shifted the dose–response curve for Oxo-M inhibition of $I_{K(M)}$, they did not completely prevent inhibition by this mAChR agonist. This partly results from the variable response between neurons expressing the $G\alpha_q$ antisense: some cells display very little inhibition when Oxo-M is applied, whereas others resemble uninjected neurons and robust inhibitions are observed (Fig. 6). This finding mirrors the results obtained when antibodies directed against $G\alpha_{q/11}$ were directly injected into SCG neurons. In these neurons there was an overall reduction in mean inhibition but a wide range of responses, from no reduction to total suppression in individual cells (Caulfield et al., 1994; Brown et al., 1995). The variability seen with the $G\alpha_q$ antisense plasmids may most reasonably be attributed to a variable reduction in endogenous $G\alpha_q$ protein. Although the participation of additional G-proteins cannot be totally excluded, this seems less likely because one would then have to postulate that the component of inhibition mediated by G_q varied greatly from one cell to another in an apparently arbitrary manner. Certainly, if another G-protein is involved, it must be insensitive to PTX (Fig. 5*D*), and it cannot be G_{11} because (1) $G\alpha_{11}$ antisense plasmids were unable to alter $I_{K(M)}$ inhibition and (2) the $G\alpha_{q/11\ common}$ antisense had no more effect than the specific $G\alpha_q$ antisense plasmids (Figs. 5*B,C*, 6).

The inequality between $G\alpha_q$ and $G\alpha_{11}$ function in these cells is somewhat surprising, because the bulk of evidence implies that $G\alpha_q$ and $G\alpha_{11}$ are indistinguishable in both receptor-coupling and effector-coupling preference. These studies have been based mainly on purified protein in cell-free systems (Taylor et al., 1991) or cloned wild-type and constitutively active proteins expressed in cell lines (Aragay et al., 1992; Wu et al., 1992a,b; Hepler et al., 1993). Studies using antisense oligonucleotides, however, have implicated (1) both $G\alpha_q$ and $G\alpha_{11}$ in M1 mAChR activation of PLC-β in RBL-2H3 cells (Dippel et al., 1996) but(2) neuromedin B receptor activation of PLC occurring via $G\alpha_q$, and not $G\alpha_{11}$, in *Xenopus* oocytes (Shapira et al., 1994). Furthermore, in both rat myocytes and *Xenopus* oocytes, endogenous $G\alpha_q$ and $G\alpha_{11}$ ap-

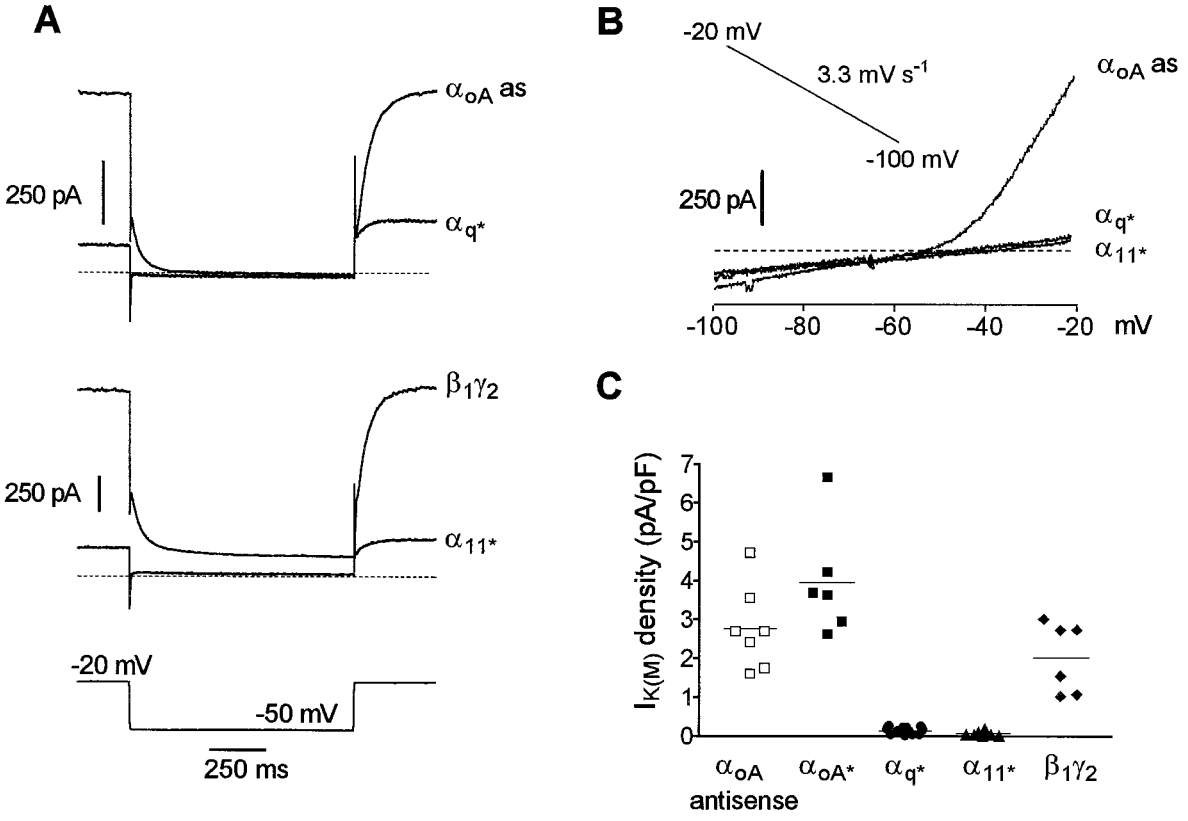

Figure 8. Expression of GTPase-deficient forms of $G\alpha_q$ and $G\alpha_{11}$ tonically inhibits $I_{K(M)}$, whereas $\beta_1\gamma_2$ dimers have no effect. *A*, Representative waveforms from cells in *C*. Neurons expressing constitutively active $G\alpha_{q^*}$ or $G\alpha_{11^*}$ have very little holding current at −20 mV and no $I_{K(M)}$ deactivation relaxation in response to a −30 mV voltage step (*bottom trace*). $I_{K(M)}$ is normal in cells expressing $\beta_1\gamma_2$ dimers compared with injected neurons (e.g., $G\alpha_{oA}$ antisense-expressing cells). Waveforms are the average of three traces, and the *dotted line* represents 0 pA. *B*, Current–voltage curves in response to a voltage ramp from −20 to −100 mV at 3.3 mV/sec (displayed in *insert*) in $G\alpha_{oA}$ antisense-expressing cells and neurons expressing $G\alpha_{q^*}$ or $G\alpha_{11^*}$. *I–V* plot is in reverse direction from the ramp applied, and the *dotted line* represents 0 pA. These traces have not been leak-subtracted; leak current in the $G\alpha_{q^*}$ and $G\alpha_{11^*}$ cells is less than in the $G\alpha_{oA}$ antisense-expressing neurons. *C*, Scatter-plot of $I_{K(M)}$ densities in $G\alpha_{oA}$ antisense-expressing cells and cells expressing $G\alpha_{oA^*}$, $G\alpha_{q^*}$, $G\alpha_{11^*}$, and $\beta_1\gamma_2$ dimers. *Horizontal lines* represent means of each group. $G\alpha_{q^*}$ ($n = 13$) and $G\alpha_{11^*}$ ($n = 8$) are significantly different from $G\alpha_{oA}$ antisense-expressing neurons ($n = 7$) ($p < 0.001$ and $p < 0.001$, respectively), $G\alpha_{oA^*}$-expressing neurons ($n = 6$) ($p < 0.001$ and $p < 0.001$, respectively), and $\beta_1\gamma_2$ dimer-expressing neurons ($n = 6$) ($p < 0.01$ and $p < 0.01$, respectively).

pear to have distinctly different functions after thyrotropin-releasing hormone or α_1-adrenergic receptor activation (Lipinsky et al., 1992; Macrez-Leprêtre et al., 1997). These studies, and our results, suggest that the coupling preferences suggested by *in vitro* and overexpression studies may not reflect those found in native cells.

The lack of involvement of $G\alpha_{11}$ in mediating $I_{K(M)}$ inhibition does not seem to arise from an inability of the subunit to couple to appropriate effector systems, because both $G\alpha_{11^*}$ and $G\alpha_{q^*}$ virtually abolished $I_{K(M)}$. It is more likely, therefore, that the M1 muscarinic receptor preferentially links to $G\alpha_q$, rather than $G\alpha_{11}$, in these neurons. Because studies with recombinant $G\alpha_q$ and $G\alpha_{11}$ have demonstrated that both of these subunits are capable of coupling to M1 receptors (Nakamura et al., 1995), differential receptor coupling might arise from a greater abundance of $G\alpha_q$ relative to $G\alpha_{11}$. Lower levels of $G\alpha_{11}$, relative to $G\alpha_q$, have been observed in most cell lines (Milligan et al., 1993) and all regions of brain examined (Milligan, 1993), and Western blots from whole ganglia indicate that these proteins may not be equally expressed in SCG (Caulfield et al., 1994). Alternatively, differences in membrane compartmentalization of $G\alpha_q$ and $G\alpha_{11}$ could result in differential access to the receptor, as has been suggested for $G\alpha_i$ and $G\alpha_o$ (Neubig, 1994; Gudermann et al., 1996). Membrane association of $G\alpha_q$ and $G\alpha_{11}$ is primarily determined by their N-terminal regions where the palmitoylation sites and the regions essential for $\beta\gamma$ subunit interaction are situated (Conklin and Bourne, 1993; Milligan et al., 1995; Hepler et al., 1996). Because this region also contains the greatest amino acid diversity between $G\alpha_q$ and $G\alpha_{11}$ (Strathmann and Simon, 1990), it is possible that they may differ in their membrane association or distribution. Recent work by Umemori et al. (1997) indicates that $G\alpha_q$ and $G\alpha_{11}$ can undergo phosphorylation by tyrosine kinase after M1 mAChR activation and that this is required for activation of the subunits and leads to disassociation of the receptor–G-protein complex. A final possibility, therefore, could be that the phosphorylation states of $G\alpha_q$ and $G\alpha_{11}$ in SCGs may differ, thereby altering their ability to interact with the receptor. Nevertheless, the effectiveness with which the GTPase-resistant $G\alpha_{11}$ inhibits $I_{K(M)}$ leaves open the possibility that $G\alpha_{11}$ may mediate PTX-insensitive inhibition of $I_{K(M)}$ via other receptors such as angiotensin II (Shapiro et al., 1994) or bradykinin (the effect of which is also inhibited by the $G\alpha_{q/11}$ antibody) (Jones et al., 1995).

The strong suppression of $I_{K(M)}$ after overexpression of GTPase-resistant α_q (and α_{11}), but not α_{oA}, suggests that inhibition might well be mediated by dissociated GTP-bound α_q

subunits but does not, of itself, exclude the possibility that $\beta\gamma$ subunits released from the endogenous $\alpha\beta\gamma$ heterotrimer might be the physiological mediator of inhibition. However, this possibility seems unlikely for two reasons. First, co-overexpression of β_1 with γ_2 subunits did not significantly reduce $I_{K(M)}$ (Fig. 8). In parallel experiments, this procedure effectively inhibited the N-type voltage-gated Ca^{2+} current in these neurons (Delmas et al., 1998a), an inhibitory process considered from previous work to be driven by free $\beta\gamma$ subunits (Ikeda, 1996; Herlitze et al., 1996). Second, muscarinic inhibition of $I_{K(M)}$ was unaffected in neurons injected with 200 μg/ml of a construct expressing the C-terminal sequence of βARK1 (also known as GRK2), which binds and sequesters free $\beta\gamma$ subunits (Koch et al., 1994) (Fig. 7). Again, in parallel experiments, this concentration of the βARK1 construct reduced I_{Ca} inhibition by noradrenaline and Oxo-M (Delmas et al., 1998a,b). Although increasing the plasmid concentration (to 400 μg/ml) did attenuate $I_{K(M)}$, this might be a nonspecific effect. We cannot exclude the possibility that the $\beta\gamma$ dimer associated with $G\alpha_q$ might have a low affinity for the βARK1 $\beta\gamma$-binding domain [for instance, β_3 subunits cannot interact with βARK1 (Daaka et al., 1997)]. However, $G\alpha_q$ and $G\alpha_{11}$ can form heterotrimers with $\beta_1\gamma_2$ dimers (Nakamura et al., 1995), and βARK1 abolishes muscarinic-activated calcium release in *Xenopus* oocytes, a response involving both $G\alpha_q$ and $G\alpha_{11}$ (Stehno-Bittel et al., 1995).

Hence, and in conclusion, our results suggest that the pathway involved in muscarinic inhibition of $I_{K(M)}$ in rat SCG neurons requires $G\alpha_q$ but not $G\alpha_{11}$ and that it is the α subunit of the heterotrimer G_q, rather than the $\beta\gamma$ dimer, that acts as the primary transducer. To this extent, they also provide further evidence that coupling between receptors and G-proteins in neurons is highly specific and more subtle than experiments with reconstituted subunits might indicate.

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