Muscarinic M-current inhibition via $G_{\alpha q/11}$ and α-adrenoceptor inhibition of Ca^{2+} current via $G_{\alpha o}$ in rat sympathetic neurones

Malcolm P. Caulfield, Susan Jones, Yvonne Vallis, Noel J. Buckley, Gun-Do Kim*, Graeme Milligan* and David A. Brown

*Wellcome Laboratory for Molecular Pharmacology, Department of Pharmacology, University College London, Gower Street, London WC1E 6BT and *Molecular Pharmacology Group, Departments of Biochemistry and Pharmacology, University of Glasgow, Glasgow G12 8QQ*

1. Microinjection of selective antibodies into superior cervical ganglion (SCG) neurones has identified the G-protein α-subunits mediating muscarinic receptor inhibition of M-type K^+ current ($I_{K(M)}$) and α-adrenoceptor inhibition of Ca^{2+} current (I_{Ca}).

2. Antibodies specific for $G_{\alpha q/11}$, but not those for $G_{\alpha o}$, reduced M-current inhibition by the muscarinic agonist oxotremorine-M, whereas anti-$G_{\alpha o}$ antibodies, but not anti-$G_{\alpha q/11}$ or anti-$G_{\alpha i1-3}$ antibodies, reduced calcium current inhibition by noradrenaline.

3. Immunoblots with specific anti-G-protein antibodies demonstrated the presence of both $G_{\alpha q}$ and $G_{\alpha 11}$, while $G_{\alpha o1}$ (but virtually no $G_{\alpha o2}$) was present.

4. We conclude that M_1 muscarinic receptor inhibition of $I_{K(M)}$ is transduced by $G_{\alpha q}$ and/or $G_{\alpha 11}$, and that $G_{\alpha o}$ transduces α-adrenoceptor inhibition of I_{Ca}.

In neurones of rat superior cervical ganglion (SCG), muscarinic receptors of the M_1 subtype couple to inhibit the M-type K^+ current ($I_{K(M)}$; Marrion, Smart, Marsh & Brown, 1989), while α_2-adrenoceptor activation inhibits voltage-gated Ca^{2+} currents (I_{Ca}; Galvan & Adams, 1982). The range of G-proteins expressed in these neurones and the precise identity of the G-proteins mediating these responses remains to be determined; however, there is some information about the nature of receptor–G-protein coupling. Muscarinic $I_{K(M)}$ suppression in SCG neurones is mediated by an as yet unidentified pertussis toxin (PTX)-insensitive G-protein (Brown, Marrion & Smart, 1989), probably via the action of a diffusible second messenger (Selyanko, Stansfeld & Brown, 1992). On the other hand, noradrenaline inhibition of I_{Ca} is through a rapid pathway not involving a second messenger (Bernheim, Beech & Hille, 1991) and a PTX-sensitive G-protein plays a major role in transducing this response (Beech, Bernheim & Hille, 1992). This suggests that the G_i or G_o class of G-proteins may be involved. There is evidence from other systems that G_o rather than G_i mediates transmitter inhibition of I_{Ca} (McFadzean, Mullaney, Brown & Milligan, 1989; Kleuss, Hescheler, Ewel, Rosenthal, Schultz & Wittig, 1991; Menon-Johannson, Berrow & Dolphin, 1993), so one or more of the subtypes of G_o seem likely candidates to couple α_2-adrenoceptors to inhibit I_{Ca} channels. For muscarinic receptor inhibition of $I_{K(M)}$, potential PTX-insensitive G-proteins which could transduce the response are the five known members of the G_q family, G_{12} or G_{13} and G_z (see Simon, Strathmann & Gautam, 1991).

Neurotransmitter receptors are thought to couple to the carboxy-terminus of G-protein α-subunits, and polyclonal antibodies raised against C-terminal peptide sequences of different G_α subunits have been shown to functionally antagonize neurotransmitter receptor modulation of ion channel currents (e.g. anti-$G_{\alpha o}$: McFadzean *et al.* 1989; Menon-Johannson *et al.* 1993; anti-$G_{\alpha q/11}$: Wilk-Blaszczak, Gutowski, Sternweis & Belardetti, 1994). In this paper, we show that microinjection into SCG neurones of specific antibodies raised against the C-terminal decapeptide sequence common to $G_{\alpha q}$ and $G_{\alpha 11}$ (but not antibodies against $G_{\alpha o}$) attenuate muscarinic receptor inhibition of $I_{K(M)}$, while antibodies against a similar region of $G_{\alpha o}$ (but not antibodies against $G_{\alpha i1-3}$ or $G_{\alpha q/11}$) reduce α-adrenoceptor inhibition of I_{Ca}. $G_{\alpha q}$, $G_{\alpha 11}$ and $G_{\alpha o1}$ were detected in SCG neurones by immunoblotting with specific antibodies. $G_{\alpha o2}$ was barely detectable. From this, we conclude that $G_{\alpha q}$ and/or $G_{\alpha 11}$ mediates muscarinic $I_{K(M)}$ inhibition, while inhibition of I_{Ca} by noradrenaline involves $G_{\alpha o}$.

METHODS

Cell culture

Superior cervical ganglia were isolated from rats (15–19 days old; killed by CO_2 asphyxiation) and were dissociated and cultured on laminin-coated plastic dishes, as described previously (see Selyanko *et al.* 1992). The dishes were marked on the underside with a grid of 1 × 1 mm squares, to help with localization of antibody-injected cells. For recording I_{Ca}, neurones were cultured for 1 day, after which they were resuspended and replated (to remove processes and thereby improve voltage clamp). Recordings were made at least 5 h after replating. During all recordings, cells were superfused at room temperature (20–26 °C) with a modified Krebs solution containing (mM): NaCl, 120; KCl, 3; $CaCl_2$, 2·5; $MgCl_2$, 1·2; $NaHCO_3$, 23; glucose, 11; Hepes, 5; tetrodotoxin, 0·0005, and bubbled with a 95 % O_2–5 % CO_2 mixture. The pH of this solution was 7·36.

PTX-catalysed ADP ribosylation of G-proteins

Cultured SCG neurones (untreated, or pretreated with 500 ng ml^{-1} PTX for 15–18 h) were washed in phosphate-buffered saline (PBS), suspended in 500 μl of 10 mM Tris-HCl–0·1 mM EDTA, pH 7·5 (Tris–EDTA) and homogenized with fifty strokes with a Teflon–glass homogenizer. The samples were then centrifuged at 150000 *g* for 10 min, the pellet resuspended in 50 μl Tris–EDTA and aliquots (20 μl) were then subjected to PTX-catalysed [^{32}P]ADP ribosylation for 2 h (37 °C). The assay mixture contained 2 μCi [^{32}P]nicotinamide adenine dinucleotide, 20 mM thymidine, 0·1 mM GTP, 250 mM sodium phosphate (pH 7·0), 1 mM ATP, 20 mM arginine hydrochloride and 1·5 μg ml^{-1} thiol-activated PTX. The assays were terminated by precipitation with sodium deoxycholate and trichloroethanoic acid. Samples were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (10 % w/v acrylamide) and autoradiographs made by exposure for 48 h.

Immunoblots

Antisera were generated in New Zealand White rabbits, as described previously (Goldsmith *et al.* 1987), using conjugates of keyhole limpet haemocyanin and synthetic decapeptides corresponding to the carboxy terminal of G-protein α-subunits. Cultured SCG neurones were washed twice with PBS and pelleted (at 500 *g*), then resuspended in Laemmli's sample buffer for resolution of G_α proteins using the SDS–PAGE system (12·5 % acrylamide, 0·0625 % bis-acrylamide, containing a linear gradient of 4–8 M urea) described by Mullaney, Mitchell, McCallan, Buckley & Milligan (1993). Following transfer to nitrocellulose, the cells were immunoblotted using an anti-$G_{\alpha q/11}$ antiserum (CQ2, 1:200 dilution: Mitchell, Mullaney, Godfrey, Arkinstall, Wakelam & Milligan, 1991), an anti-$G_{\alpha o1/2}$ antiserum (OC2, 1:1000 dilution), or an anti-$G_{\alpha i1/2}$ antiserum (SG1, 1:400).

Injection of antisera

Neurones were injected with either anti-G-protein antiserum or rabbit antiserum raised against human brain glial fibrillary acidic protein (anti-GFAP; Sigma Chemical Co., UK). The anti-G-protein antisera used were those described above, and also an antiserum (I3C) raised against the C-terminal decapeptide sequence of $G_{\alpha 1\text{-}3}$ (KNNLKECGLY), as described by Goldsmith *et al.* (1987). Injections were made from high resistance (> 30 MΩ) electrodes, and cell penetration was apparent from the appearance of a standing negative voltage while in 'bridge' recording mode. Antiserum was expelled by application of gentle pressure to the back of the electrode with a syringe. Recordings were made at least 2 h after injection of antiserum.

$I_{K(M)}$ recording

Whole-cell $I_{K(M)}$ currents were recorded using the nystatin perforated patch method (Horn & Marty, 1988) from neurones which had been cultured for 2 days. Patch pipettes (5–8 MΩ) were filled by dipping the tip into a filtered solution containing (mM): potassium acetate, 80; KCl, 30; $MgCl_2$, 3; Hepes, 40 (adjusted to pH 7·4 and 290 mosmol l^{-1}), for 15–60 s, after which the pipette was back-filled with the above solution containing 0·25 mg ml^{-1} nystatin. After achieving high resistance (> 2 GΩ) seals, access resistances after nystatin permeabilization were < 30 MΩ. Neurones were voltage clamped at about −25 mV using a switching amplifier (Axoclamp 2A, Axon Instruments, Foster City, CA, USA; switching frequency 2–4 kHz) and $I_{K(M)}$ was recorded as the slowly developing inward deactivation relaxation during 1 s jumps to a command potential of about −55 mV (Fig. 2). Muscarinic inhibition of $I_{K(M)}$ was measured after perfusion with a solution containing the muscarinic agonist oxotremorine methiodide (Oxo-M, 300 nM; Semat, St Albans, UK). We only analysed data from cells showing recovery of $I_{K(M)}$ of at least 60 % from agonist-inhibited levels (usually within 5 min).

I_{Ca} recording

Voltage-gated Ca^{2+} currents were recorded in whole-cell mode, using patch pipettes filled with a solution containing (mM): CsCl, 13; caesium acetate, 120; Hepes, 10; Cs-EGTA, 3; NaATP, 2; NaGTP, 0·5; $MgCl_2$, 4; adjusted to pH 7·4 with CsOH, and to 290 mosmol l^{-1}. Neurones were voltage clamped (Axoclamp 2A) at −80 mV and I_{Ca} were evoked every 60 s by stepping for 50 ms to +10 mV. A pulse to −40 mV immediately preceding the command step served to reduce capacity transients (Bernheim *et al.* 1991). I_{Ca} amplitude was estimated by digitally subtracting the outward current (using pCLAMP software; Axon Instruments) remaining during the same voltage step in the presence of Krebs solution in which $CaCl_2$ had been replaced with $CoCl_2$. Inhibition of I_{Ca} was measured 1 min after the normal perfusion solution was changed to one containing noradrenaline (which had no effect on the outward current in the presence of Co^{2+}; $n = 3$, data not shown). Data were only included from cells in which recovery from noradrenaline inhibition was to > 90 % of the control current.

Visualization of injected antibody

After a series of injections and recordings, the Krebs solution in the dish was replaced by ice-cold 0·1 M PBS containing 4 % bovine serum albumin (PBS–BSA). After 2 min, the cells were permeabilized and fixed for 15 min at −20 °C in 5 % acetic acid in ethanol. The dish was then washed 3 times with PBS–BSA, and the solution was finally replaced with 1 ml of a 1:100 solution (in PBS–BSA) of fluorescein isothiocyanate-labelled sheep anti-rabbit IgG antibody (Serotec, Oxford, UK). After incubation for 30 min at room temperature, unbound second layer antibody was removed by three washes with PBS–BSA, and the preparation was mounted in Citifluor (Citifluor,

Guildford, UK) under a glass coverslip. The identified injected (and recorded) cells were then checked for antibody loading under a fluorescence microscope.

RESULTS

Expression of G-proteins in SCG neurones

Resolution of membranes of SCG neurones by SDS–PAGE, followed by immunoblotting with specific anti-G-protein antisera showed very strong bands for $G_{\alpha q}$, $G_{\alpha 11}$, (Fig. 1*A*), $G_{\alpha o1}$ (Fig. 1*Ba*) and $G_{\alpha i2}$ (Fig. 1*Bb*). Immunoblots showed very faint bands of $G_{\alpha o2}$ (Fig. 1*Ba*) and $G_{\alpha i1}$ (Fig. 1*Bb*) proteins, indicating that the G_o and G_i subtypes expressed in SCG neurones are almost exclusively $G_{\alpha o1}$ and $G_{\alpha i2}$, respectively. The ability of the immunoblotting procedure to distinguish G_i and G_o isoforms was verified by detection of G_{i1} and G_{i2} (in membranes of rat frontal cortex; Fig. 1*Bb*), and G_{o1}, G_{o2} and G_o* (in membranes of NG108-15 neuroblastoma cells; Fig. 1*Ba*; see Mullaney & Milligan, 1990). Reverse transcription/polymerase chain reaction analysis, using specfic $G_{\alpha i3}$ primers, demonstrated the presence of $G_{\alpha i3}$ transcripts in cultures of SCG neurones (N. J. Buckley, unpublished observations).

PTX pretreatment of SCG neurones (500 ng ml^{-1} for 15–18 h) achieved almost complete ADP ribosylation of susceptible G-proteins, as measured by inhibition of incorporation of [^{32}P]-labelled nicotinamide adenine dinucleotide (Fig. 1*C*).

Anti-$G_{\alpha q/11}$ antibodies reduce muscarinic inhibition of $I_{K(M)}$

To test the effects of antibody injection or PTX pretreatment, we chose to use 300 nM Oxo-M as a test concentration, since any change in efficiency of receptor–G-protein transduction would have been detectable by the submaximal response to this agonist concentration (in five experiments, the mean concentration of Oxo-M producing half-maximal $I_{K(M)}$ inhibition was 166 nM, with a Hill slope of 1·3). In order to ensure that we measured transmitter responses only from neurones which had been successfully injected with antibody, we routinely stained cells to demonstrate antibody loading once electrophysiological experiments were completed. Figure 2 shows a neurone which was injected with anti-$G_{\alpha q/11}$ antibody and then, after recording $I_{K(M)}$ and its inhibition by Oxo-M, stained with a second fluorescein-tagged antibody. The bright fluorescence in the antibody-injected neurone, but not in the uninjected neurone, was taken to indicate loading with anti-G-protein (or control) antibody. About 90 % of anti-

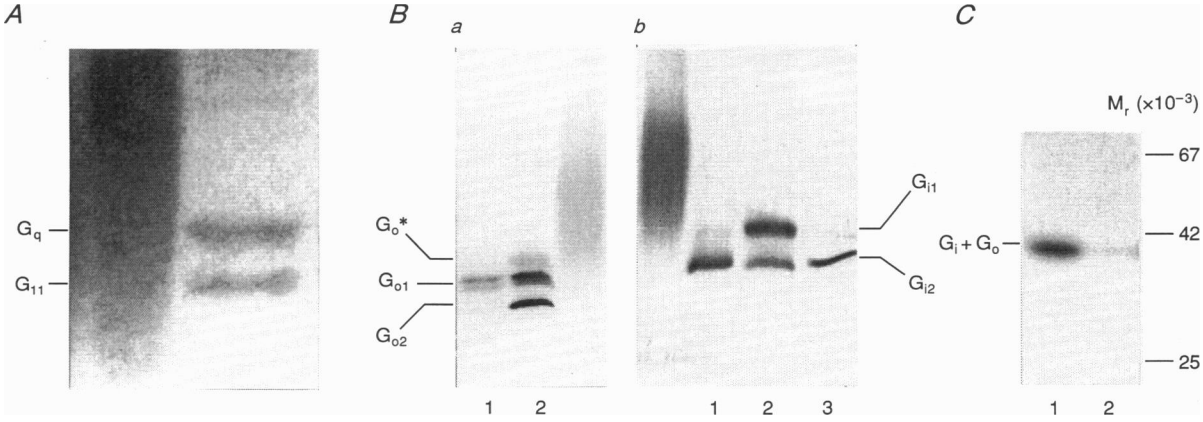

Figure 1. Expression of G-proteins by SCG neurones, and ADP ribosylation of susceptible G-proteins by PTX

A, immunological detection of the co-expression of $G_{\alpha q}$ and $G_{\alpha 11}$ in SCG neurones. The sample in the left-hand lane of the immunoblot is prestained lactic dehydrogenase (LDH; Sigma, UK), which we have noted to migrate in a similar position to $G_{\alpha q}$ and $G_{\alpha 11}$ in such gels, and was used as a marker for the migration of the G-proteins. Bands showing $G_{\alpha q}$ and $G_{\alpha 11}$ are in the right-hand lane. *B*, immunological detection of the co-expression of $G_{\alpha o1}$ and $G_{\alpha i2}$, and barely detectable levels of $G_{\alpha o2}$ or $G_{\alpha i1}$ in SCG neurones. *Ba*, $G_{\alpha o1}$ (but virtually no $G_{\alpha o2}$) in SCG membranes is shown in lane 1 of the immunoblot. Lane 2 shows $G_{\alpha o1}$, $G_{\alpha o2}$ and G_o* in membranes (100 μg) from NG108-15 cells. The LDH size marker was run in the far right lane. *Bb*, $G_{\alpha i2}$ (with barely detectable expression of $G_{\alpha i1}$) in SCG neurones is shown in lane 1 of the immunoblot, while lane 2 shows predominant expression of both $G_{\alpha i1}$ and $G_{\alpha i2}$ in rat brain frontal cortex membranes (25 μg), and lane 3 shows $G_{\alpha i2}$ (but no detectable $G_{\alpha i1}$) in NG108-15 cell membranes (50 μg). The LDH size marker is in the far left lane. *C*, PTX-catalysed [^{32}P]ADP ribosylation of membranes of control and PTX-treated SCG neurones. Autoradiographs show that PTX pretreatment (lane 2) caused virtually complete ADP ribosylation of the available pool of PTX-sensitive G-proteins (G_i and G_o, which cannot be distinguished because they co-migrate in this gel system). Untreated neurones (lane 1) showed good incorporation of radioactivity into these polypeptides.

body-injected neurones survived the experimental protocol and showed bright fluorescence after immunostaining. Since we were concerned that injection of antiserum might itself influence transmitter responses, neurones injected with antibody to glial fibrillary acidic protein (GFAP) were used as antibody-injected controls.

In neurones injected with antibodies raised against $G_{\alpha q/11}$, the standard deviation of Oxo-M inhibition of $I_{K(M)}$ was significantly greater ($P = 0{\cdot}02$; Bartlett's test) than in either uninjected, anti-GFAP- or anti-$G_{\alpha o}$-injected cells (Fig. 3), so statistical analyses were carried out using Dunn's non-parametric test. This showed that median inhibition of $I_{K(M)}$ in anti-$G_{\alpha q/11}$ antibody-treated neurones (32·0 %) was significantly less ($P < 0{\cdot}05$) than in anti-GFAP antibody-injected neurones, (56·0 %), uninjected neurones (59·5 %) or anti-$G_{\alpha o}$ antibody-injected neurones (63 %), respectively. In the anti-$G_{\alpha q/11}$ antibody-treated neurones, the amplitude of $I_{K(M)}$ (188 ± 19·4 pA) was not significantly different from that seen in the anti-GFAP antibody-injected neurones (174 ± 22·8 pA), the anti-$G_{\alpha o}$ antibody-treated neurones (133 ± 6·2 pA), and the uninjected neurones (159 ± 17 pA), showing that antibody injection *per se* did not alter $I_{K(M)}$. We also confirmed the previous observation (Brown *et al.* 1989) that muscarinic inhibition of $I_{K(M)}$ in PTX-treated (500 ng ml^{-1} for 15–18 h) SCG neurones (median inhibition by 300 nM Oxo-M, 56 %) did not differ from that in control cells (Fig. 3*B*).

Anti-G_o antibodies reduce α-adrenoceptor inhibition of I_{Ca}

We also tested the ability of the different G-protein antibodies to suppress α-adrenoceptor inhibition of I_{Ca} in SCG

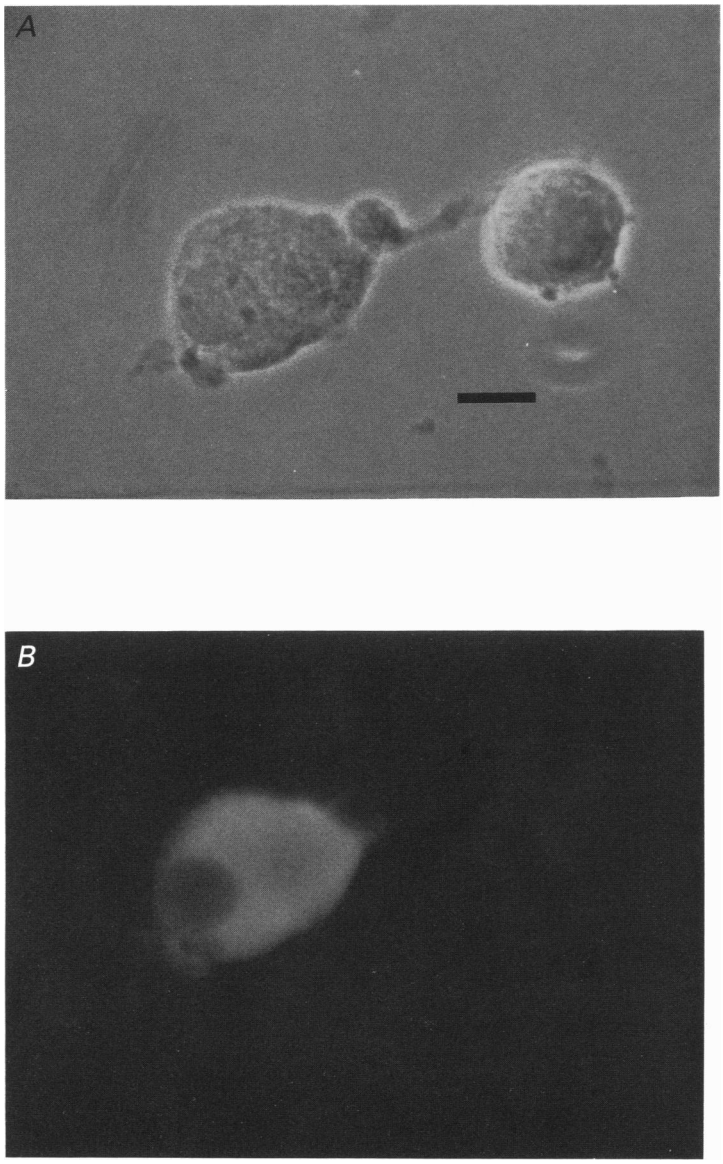

Figure 2. Labelling with a fluorescent second antibody demonstrates successful injection of an SCG neurone with anti-$G_{\alpha q/11}$ antibody
A, phase contrast photomicrograph showing a neurone injected with anti-$G_{\alpha q/11}$ antibody on the left and an uninjected neurone on the right. The scale bar represents 10 μm. *B*, fluorescence photomicrograph of the neurons in *A*. Only the antibody-injected neurone shows significant fluorescence.

neurones. Noradrenaline (1 μM) gave readily measurable, submaximal I_{Ca} inhibition (from three experiments, the mean concentration producing half-maximal response was 140 nM, while the mean Hill slope of the concentration–response relationship was 1·45). This concentration was used in subsequent experiments. In neurones which had been pre-injected with anti-$G_{\alpha o}$ antibody, inhibition of I_{Ca} by 1 μM noradrenaline was 27·0 ± 3·3 % ($n = 12$), significantly less ($P < 0·001$, ANOVA and Dunnet's test) than in uninjected (53·7 ± 2·4 %; $n = 11$) or anti-GFAP-injected cells (46·4 ± 3·1 %; $n = 9$; Fig. 4). In contrast, neurones injected with antibodies recognizing the C-terminus of $G_{\alpha q/11}$, $G_{\alpha 11/2}$ and $G_{\alpha 13}$ exhibited noradrenaline inhibition of I_{Ca} (41·1 ± 4·9 %, $n = 7$; 41·3 ± 5·4 %, $n = 9$; and 34·5 ± 3·1 %, $n = 13$, respectively) which did not differ significantly from that of anti-GFAP-injected cells (Fig. 4). Neurones injected with either control antibody (against GFAP) or anti-G-protein antibodies had mean I_{Ca} amplitudes of 0·89–0·96 nA (standard errors ranged from 0·11 to 0·22 nA). These were not different from I_{Ca} in uninjected neurones (0·96 ± 0·13 nA; ANOVA and Dunnet's test), showing that antibody injection *per se* did not alter I_{Ca}.

Treatment of neurones with PTX (500 ng ml^{-1} for 15–18 h) reduced noradrenaline I_{Ca} inhibition to 13·2 ± 1·7 % ($n = 5$); neurones incubated in a higher concentration of PTX (2·5 μg ml^{-1} for 15–18 h) showed similar responses to noradrenaline (14·8 ± 2·6 %; $n = 4$). Injection of anti-$G_{\alpha o}$ antibodies into PTX-pretreated neurones reduced the noradrenaline inhibition of I_{Ca} almost to zero (6·6 ± 1·8 %, $n = 11$; Fig. 4).

DISCUSSION

Many cells express a wide range of heterotrimeric G-proteins. We have used immunoblots with specific antisera to demonstrate expression of the G-proteins $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha o1}$ and $G_{\alpha 12}$ in SCG neurones. $G_{\alpha o2}$ and $G_{\alpha i1}$ were

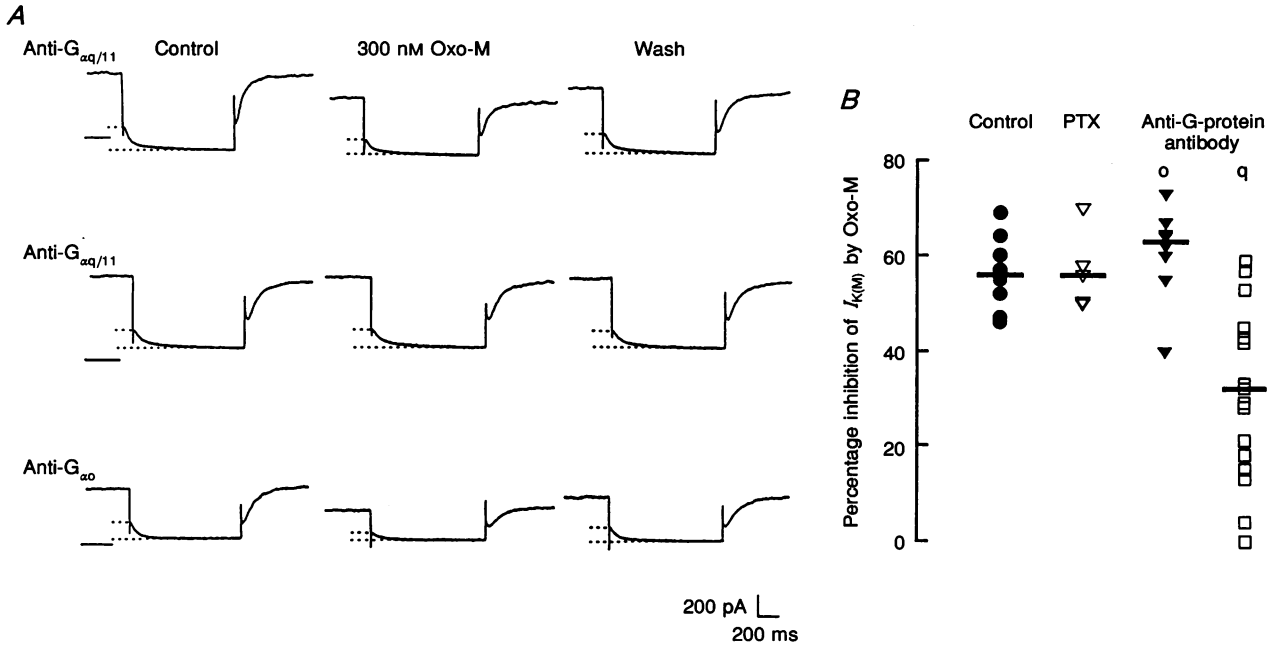

Figure 3. Anti-$G_{\alpha q/11}$ antibody injected in SCG neurones significantly reduces $I_{K(M)}$ inhibition by Oxo-M, while anti-GFAP or anti-$G_{\alpha o}$ antibody injection, or PTX pretreatment were without effect

A, representative traces showing M-current deactivation relaxations (between the dotted lines on each trace) during a 1 s hyperpolarizing step to −55 mV from a holding potential of −25 mV. Control traces are shown on the left, currents in the presence of 300 nM oxotremorine-M (Oxo-M) are in the middle, and the right-hand traces show recovery after removing the agonist. The zero current level is indicated by the continuous line. The top series of traces shows a reduction of muscarinic M-current inhibition in a neurone injected with anti-$G_{\alpha q/11}$ antibody, while the middle traces show almost complete abolition of the muscarinic response in another neurone injected with the same antibody. The bottom traces show a normal inhibition of M-current by Oxo-M in a neurone which had been injected with an anti-$G_{\alpha o}$ antibody. *B*, scatter plot showing $I_{K(M)}$ inhibition by 300 nM Oxo-M in neurones injected with anti-GFAP antibody (control; $n = 9$), neurones treated with 500 ng ml^{-1} PTX (PTX; $n = 5$), neurones injected with anti-$G_{\alpha o}$ antibody (o; $n = 10$) and anti $G_{\alpha q/11}$ antibody (q; $n = 17$). Median values are indicated by the horizontal bars.

virtually undetectable. The strong bands for $G_{\alpha o1}$ and $G_{\alpha i2}$ in the immunoblots clearly show that these are the major isoforms of $G_{\alpha o}$ and $G_{\alpha i}$ in SCG neurones. Injection of SCG neurones with selective anti-G-protein antibodies has demonstrated a divergence in coupling pathways between muscarinic M_1 receptors and inhibition of the M-type K^+ current on the one hand, and between α-adrenoceptors and inhibition of calcium currents on the other. Muscarinic inhibition of $I_{K(M)}$ was significantly reduced in neurones loaded with anti-$G_{\alpha q/11}$ antibodies, while adrenergic inhibition of I_{Ca} was diminished in cells injected with antibodies against $G_{\alpha o}$. Inhibition of either transmitter response by the antibodies was not complete and, in the case of $I_{K(M)}$ and anti-$G_{\alpha q/11}$ antibodies, varied from no inhibition to complete abolition of the response. We feel that this may result from variations in the amount of antibody injected into each cell.

Our finding that $I_{K(M)}$ inhibition is mediated by $G_{\alpha q}$ and/or $G_{\alpha 11}$ (and the lack of effect of anti-$G_{\alpha o}$ antibodies) is consistent with the PTX-insensitive muscarinic inhibition of $I_{K(M)}$ previously demonstrated in rat SCG neurones (Brown *et al.* 1989). There is evidence that muscarinic $I_{K(M)}$ inhibition in SCG neurones is mediated by a diffusible second messenger (Selyanko *et al.* 1992) and our finding that the response is transduced by $G_{\alpha q/11}$ suggests a product of phospholipase C (PLC) as a second messenger. This is because muscarinic receptor activation has been shown to increase inositol 1,4,5-trisphosphate ($InsP_3$) levels

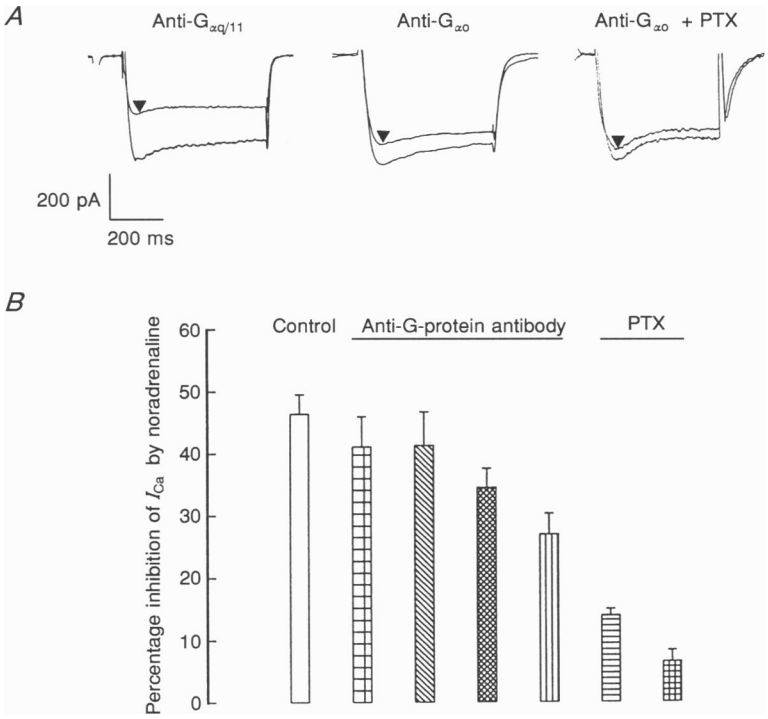

Figure 4. Anti-$G_{\alpha o}$ antibody reduces noradrenaline inhibition of I_{Ca} in SCG neurones, while anti-$G_{\alpha q/11}$, anti-$G_{\alpha i1/2}$ and anti-$G_{\alpha i3}$ antibodies are without effect

A, representative traces showing I_{Ca} (evoked by voltage steps to +10 mV from a holding potential of −80 mV), with control currents superimposed on currents reduced in the presence of 1 μM noradrenaline (▲). The left-hand traces show normal inhibition of I_{Ca} in a cell injected with an anti-$G_{\alpha q/11}$ antibody, while the middle traces show reduction of the noradrenaline response in a cell which had been injected with an anti-$G_{\alpha o}$ antibody. The right-hand traces show further reduction of the noradrenaline I_{Ca} inhibition in an anti-$G_{\alpha o}$ antibody-injected cell which had been pretreated with pertussis toxin. The inward tail current evident in the trace on the right probably results from inadequate space clamp, perhaps due to residual processes which survived the resuspension and replating procedure. *B*, histogram showing I_{Ca} inhibition by 1 μM noradrenaline in anti-GFAP (control, □; $n = 9$), anti-$G_{\alpha q/11}$ (⊞; $n = 7$), anti-$G_{\alpha i1/2}$ (▨; $n = 9$), anti-$G_{\alpha i3}$ (▩; $n = 13$) and anti-$G_{\alpha o}$ (▥; $n = 12$) antibody-injected neurones. Also shown is noradrenaline inhibition of I_{Ca} in PTX-treated neurones either uninjected or injected with anti-GFAP (▤; $n = 9$) or anti-$G_{\alpha o}$ antibody (▦; $n = 11$).

in intact rat SCG (Bone, Fretten, Palmer, Kirk & Michell, 1984) and coupling of M_1 muscarinic receptors to G_q or G_{11} can stimulate phopholipase Cβ (PLCβ) activity (Berstein *et al.* 1992). However, the identity of the second messenger involved remains elusive (see Robbins, Marsh & Brown, 1993).

In contrast to muscarinic inhibition of $I_{K(M)}$, injection of anti-$G_{\alpha q/11}$ antibodies did not affect noradrenaline inhibition of I_{Ca} and antibodies raised against the C-terminus of $G_{\alpha o1/2}$ significantly reduced the inhibitory effect of noradrenaline on I_{Ca}, pointing to $G_{\alpha o1}$ or $G_{\alpha o2}$ as a mediator. However, our finding that the predominant $G_{\alpha o}$ isoform in SCG neurones is $G_{\alpha o1}$ may indicate that this is the G-protein that couples α-adrenoceptors to I_{Ca} channels, although we cannot discount the possibility that even the small amount of $G_{\alpha o2}$ may mediate this response. In contrast, antibodies raised against the C-terminus of $G_{\alpha i1/2}$ and of $G_{\alpha i3}$ did not significantly alter adrenoceptor suppression of I_{Ca}. These negative findings could be attributed to inactivity of the antibodies, or insufficient loading of cells with antibody. However, we feel that these are unlikely explanations and that our results suggest that G_{i1-3} and $G_{q/11}$ do not transduce the noradrenaline response, for the following reasons: (1) our analysis was based solely on data from cells which had been loaded with antibodies, as evidenced by immunocytochemical visualization post-recording; (2) the antibodies raised against $G_{\alpha i1/2}$ and $G_{\alpha i3}$ which we used have been shown to functionally inhibit an action of the target G-proteins (i.e. receptor-mediated stimulation of GTPase: see McClue & Milligan, 1991); and (3) the anti-$G_{\alpha q/11}$ antibody was clearly effective in inhibiting muscarinic modulation of $I_{K(M)}$.

The conclusion that $G_{\alpha o}$, but not $G_{\alpha i}$, couples α-adrenoceptors to I_{Ca} is paralleled by similar findings with selective antibodies in NG108-15 neuroblastoma cells (McFadzean *et al.* 1989). The use by Kleuss *et al.* (1991) of antisense oligonucleotide sequences in a rat pituitary cell line has identified $G_{\alpha o1}$ as the mediator of L-type I_{Ca} inhibition by muscarinic receptors, so it appears that α-adrenergic receptors (our data) may couple to I_{Ca} channels through the same G-protein as muscarinic receptors.

The PTX-insensitive portion of the α-adrenoceptor inhibition of I_{Ca} was also noted in SCG neurones by Beech *et al.* (1992), who concluded that a PTX-insensitive G-protein can inhibit I_{Ca} channels. We felt that the apparent PTX-insensitive response did not result from inadequate ADP ribosylation of $G_{\alpha o1}$ by PTX, as this was nearly total even with 500 ng ml^{-1} PTX. Also, a higher concentration of PTX (2·5 μg ml^{-1}) inhibited the response to the same extent as 500 ng ml^{-1} PTX. Given this, we expected that anti-$G_{\alpha o}$ antibodies should produce no further reduction of α-adrenoceptor response in PTX-treated cells, so we were interested to find that noradrenaline inhibition of I_{Ca} was virtually eliminated by a combination of PTX pretreatment and injection of anti-$G_{\alpha o}$ antibody. It is possible that the residual response in PTX-treated neurones is mediated by a population of G-protein which is not ADP ribosylated, for example, any $G_{\alpha o}$ which remains dissociated from $\beta\gamma$ during the PTX treatment. Another possibility is that agonist-occupied receptors can still partially activate either ADP ribosylated or antibody-bound $G_{\alpha o1}$ subunits, and only a combination of ADP ribosylation by PTX together with antibody can completely prevent receptor activation of $G_{\alpha o}$.

In conclusion, the data we have obtained identify further key elements in the known complex transduction pathways between M_1 muscarinic receptors and M-type K^+ channels, and between α-adrenoceptors and Ca^{2+} channels in SCG neurones. Fast inhibition of I_{Ca} is probably the result of a direct action of activated $G_{\alpha o}$ subunits on 'N-type' Ca^{2+} channels, while muscarinic receptor inhibition of $I_{K(M)}$ operates through a $G_{\alpha q/11}$-mediated indirect mechanism involving a second messenger (Selyanko *et al.* 1992).

REFERENCES

Beech, D. J., Bernheim, L. & Hille, B. (1992). Pertussis toxin and voltage dependence distinguish multiple pathways modulating calcium channels of rat sympathetic neurons. *Neuron* **8**, 97–106.

Bernheim, L., Beech, D. J. & Hille, B. (1991). A diffusible second messenger mediates one of the pathways coupling receptors to calcium channels in rat sympathetic neurons. *Neuron* **6**, 859–867.

Berstein, G., Blank, J., Smrcka, A., Higashijima, T., Sternweis, P., Exton, J. & Ross, E. (1992). Reconstitution of agonist-stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis using purified m1 muscarinic receptor, Gq/11 and phospholipase C-β1. *Journal of Biological Chemistry* **267**, 8081–8088.

Bone, E. A., Fretten, P., Palmer, S., Kirk, C. J. & Michell, R. H. (1984). Rapid accumulation of inositol phosphates in isolated rat superior cervical ganglia exposed to V1 vasopressin and muscarinic cholinergic stimuli. *Biochemical Journal* **22**, 803–811.

Brown, D. A., Marrion, N. V. & Smart, T. G. (1989). On the transduction mechanism for muscarine-induced inhibition of M-current in cultured rat sympathetic neurons. *Journal of Physiology* **413**, 469–488.

Galvan, M. & Adams, P. R. (1982). Control of calcium current in rat sympathetic neurons by noradrenaline. *Brain Research* **244**, 135–144.

Goldsmith, P., Gierschik, P., Milligan, G., Unson, C. G., Vinitsky, R., Malech, H. & Spiegel, A. M. (1987). Antibodies directed against synthetic peptides distinguish between GTP-binding proteins in neutrophil and brain. *Journal of Biological Chemistry* **262**, 14683–14688.

Horn, R. & Marty, A. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. *Journal of General Physiology* **92**, 145–159.

Kleuss, C., Hescheler, J., Ewel, C., Rosenthal, W., Schultz, G. & Wittig, B. (1991). Assignment of G-protein subtypes to specific receptors inducing inhibition of calcium currents. *Nature* **353**, 43–48.

McClue, S. J. & Milligan, G. (1991). Molecular interaction of the human α_2-C10-adrenergic receptor, when expressed in Rat-1 fibroblasts, with multiple pertussis toxin-sensitive guanine nucleotide-binding proteins: studies with site-directed antisera. *Molecular Pharmacology* **40**, 627–632.

McFadzean, I., Mullaney, I., Brown, D. A. & Milligan, G. (1989). Antibodies to the GTP binding protein, G_o, antagonize noradrenaline-induced calcium current inhibition in NG108-15 hybrid cells. *Neuron* **3**, 177–182.

Marrion, N. V., Smart, T. G., Marsh, S. J. & Brown, D. A. (1989). Muscarinic suppression of the M-current in the rat sympathetic ganglion is mediated by receptors of the M_1-subtype. *British Journal of Pharmacology* **98**, 557–573.

Menon-Johansson, A. S., Berrow, N. & Dolphin, A. C. (1993). G_o transduces $GABA_B$ receptor modulation of N-type calcium channels in cultured dorsal root ganglion neurones. *Pflügers Archiv* **193**, 1–9.

Mitchell, F. M., Mullaney, I., Godfrey, P. P., Arkinstall, S. J., Wakelam, M. J. O. & Milligan, G. (1991). Widespread distribution of $G_{q\alpha}/G_{11\alpha}$ detected immunologically by an anti-peptide antiserum directed against the predicted C-terminal decapeptide. *FEBS Letters* **287**, 171–174.

Mullaney, I. & Milligan, G. (1990). Identification of two distinct isoforms of the guanine nucleotide binding protein, G_o in neuroblastoma × glioma hybrid cells: Independent regulation during cyclic AMP-induced differentiation. *Journal of Neurochemistry* **55**, 1890–1898.

Mullaney, I., Mitchell, F. M., McCallan, J. F., Buckley, N. J. & Milligan, G. (1993). The human muscarinic m_1 acetylcholine receptor, when expressed in CHO cells, activates and downregulates both $G_{q\alpha}$ and $G_{11\alpha}$ equally and non-selectively. *FEBS Letters* **324**, 241–245.

Robbins, J., Marsh, S. J. & Brown, D. A. (1993). On the mechanism of M-current inhibition by muscarinic m1 receptors in DNA-transfected rodent neuroblastoma × glioma cells. *Journal of Physiology* **469**, 153–178.

Selyanko, A. A., Stansfeld, C. E. & Brown, D. A. (1992). Closure of potassium M-channels by muscarinic acetylcholine-receptor stimulants requires a diffusible messenger. *Proceedings of the Royal Society* B **250**, 119–125.

Simon, M. I., Strathmann, M. P. & Gautam, N. (1991). Diversity of G proteins in signal transduction. *Science* **252**, 802–808.

Wilk-Blaszczak, M. A., Gutowski, S., Sternweis, P. C. & Belardetti, F. (1994). Bradykinin modulates potassium and calcium currents in neuroblastoma hybrid cells via different pertussis toxin-insensitive pathways. *Neuron* **12**, 119–126.

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