Receptors couple to L-type calcium channels via distinct G_o proteins in rat neuroendocrine cell lines

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1. The present study examines the hypothesis of G protein subtype selectivity in receptor-induced inhibition of calcium channel currents (I_{Ca}) in the insulin-secreting RINm5F and pituitary GH_3 rat cell lines. Specificity of receptor coupling to G proteins was studied by infusion of purified $G\alpha$ isoforms into cells via a patch pipette.

2. In RINm5F cells, the neuropeptide galanin inhibited dihydropyridine (DHP)- and ω-conotoxin-sensitive components of I_{Ca} and slowed down their activation kinetics. In GH_3 cells, DHP-sensitive I_{Ca} was inhibited by galanin, as well as by somatostatin and carbachol. Agonist-induced I_{Ca} inhibition was suppressed by pertussis toxin (PTX) pretreatment of the cells. In PTX-pretreated cells of either cell line, the response to galanin was restored only by the $G\alpha_{o1}$ subunit. Following PTX treatment of GH_3 cells, only the $G\alpha_{o1}$ subunit restored carbachol-induced inhibition of I_{Ca}, whereas only the $G\alpha_{o2}$ subunit restored somatostatin-induced inhibition of I_{Ca}. G_i subtypes had no effect on I_{Ca} inhibition.

3. Both cell lines expressed two distinct immunoreactive G_o proteins. Whereas in RINm5F cell membranes G_{o1} was found to be the predominant isoform, we detected more G_{o2} than G_{o1} in GH_3 cell membranes. Nevertheless, all agonists stimulated incorporation of the photoreactive GTP analogue [α-^{32}P]GTP azidoanilide into both G_o isoforms.

4. The results indicate that the same G_o subtype, i.e. G_{o1}, mediates galanin-induced inhibition of I_{Ca} in both cell lines and that the G_o subtype specificity of receptor–G protein coupling is confined to intact cells.

G protein-dependent transmembranous signalling cascades involve three independent transduction elements: heptahelical receptors, G proteins and cellular effectors (for review, see Hille, 1994; Hofman, Biel & Flockerzi 1994; Dolphin, 1995; Nürnberg, Gudermann & Schultz, 1995). Ligand binding to the cell surface receptor enables conformational changes of the receptor, thereby activating G proteins. These function as versatile switches regulating various cellular effectors. The G protein-coupled signalling complex consists of multiple branch points allowing signal amplification. For instance, one light-activated rhodopsin stimulates thousands of transducin molecules during its life-time (Hargrave, Hamm & Hofmann, 1993). In addition, signal convergence and divergence appear to be accomplished by the G protein-dependent signalling cascade, i.e. different ligand-activated heptahelical receptors co-expressed in the same cell stimulate an identical class of G proteins or different G protein isoforms which modulate the same effector system. Conversely, one G protein-coupled receptor like the thyrotrophin receptor couples to members of as much as four different G protein subfamilies, thereby regulating multiple cellular pathways concurrently (for review, see Offermanns & Schultz, 1994).

Nevertheless, an extremely high degree of specificity and selectivity of G protein coupling appears to be present (for review, see Kalkbrenner, Dippel Wittig & Schultz, 1996). Functional knock-out of G protein subunits by injection of antisense-RNA suggests specific subunit compositions of G proteins. In GH_3 cells, for example, carbachol-induced calcium current (I_{Ca}) inhibition was specifically suppressed by injection of antisense oligonucleotides directed against $G\alpha_{o1}\beta_3\gamma_4$ (Kleuss, Scherübel, Hescheler, Schultz & Wittig, 1993). In the same cells the heterotrimer $G\alpha_{o2}\beta_1\gamma_3$ was identified as mediating somatostatin-induced I_{Ca} inhibition, whereas galanin induced the same effect via $G\alpha_{o1}\beta_2\gamma_2$ and, less efficiently via $G\alpha_{o1}\beta_3\gamma_4$ (Kalkbrenner *et al.* 1995). However, while these experiments identified the predominant G protein triplet, they did not rule out participation of additional G proteins in the pertussis toxin (PTX)-sensitive inhibition of voltage-activated calcium channels. In fact, previous studies implied that somatostatin and galanin receptors couple to multiple PTX-sensitive $G_{i/o}$ proteins (Schmidt *et al.* 1991; Law, Yasuda, Bell & Reisine, 1993; Hadcock, Strnad & Eppler, 1994). Furthermore, we detected quantitative differences in the expression of G_o proteins in

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the membranes of GH_3 and RINm5F cells. This observation raised the question of whether the quantitative pattern of G protein expression in a cell influences the selectivity of a receptor–effector signalling pathway. In this study, we characterized calcium channels in GH_3 and RINm5F cells and their PTX-sensitive inhibition by G protein-coupled receptors. The receptors were uncoupled from G proteins by pretreatment with PTX. This manipulation almost completely abolished hormone-induced inhibition of L-type voltage-operated calcium channels. Subsequently, calcium currents were restored by infusion of specific $G\alpha_o$ subunits into the cells.

METHODS

Materials

Rat or pig galanin was obtained from Saxon Biochemicals (Hannover, Germany); if not specified, rat galanin was used. Carbachol, somatostatin, EGTA and BAPTA were from Sigma. ω-Conotoxin GVIA (ω-CTX GVIA), ω-conotoxin MVIIC (ω-CTX MVIIC) and ω-agatoxin IVA (ω-Aga IVA) were from Alomone Labs (Jerusalem, Israel). The dihydropyridine (+)-isradipine was supplied by Sandoz (Nürnberg, Germany). ^{35}S-GTPγS (1100–1400 Ci mmol^{-1}), [α-^{32}P]ATP (800 Ci mmol^{-1}) and [α-^{32}P]GTP (3000 Ci mmol^{-1}) were purchased from DuPont New England Nuclear (Bad Homburg, Germany). ^{32}P-NAD was prepared according to Cassel & Pfeuffer (1978), and the photoreactive GTP analogue [α-^{32}P]GTP azidoanilide was synthesized as described by Laugwitz, Spicher, Schultz & Offermanns (1994). The sources of all other reagents are reported elsewhere (Nürnberg *et al.* 1994; Obukhov *et al.* 1996). Stock solutions of galanin, somatostatin, carbachol, ω-CTX and ω-Aga IVA toxins were made in distilled water at concentrations of 0·5, 1, 10 and 10 mM, respectively. The stock solution of (+)-isradipine was made in DMSO. All stock solutions were stored in aliquots at −22 °C and diluted in external solutions E2 or E3 (see below) immediately before use.

Cell culture

Rat pituitary GH_3 and insulin-secreting RINm5F cells were obtained from the American Type Culture Collection (Rockville, MN, USA). The GH_3 cells were cultured in Ham's F-10 medium supplemented with 13% (v/v) horse serum, 2% fetal calf serum, 4 mM L-glutamine, non-essential amino acids, 0·05 mg ml^{-1} streptomycin and 50 units ml^{-1} penicillin in an atmosphere of 7% CO_2 in air. The RINm5F cells were cultured using RPMI-1640 medium containing 8% fetal calf serum, 18 mM L-glutamine, 0·05 mg ml^{-1} streptomycin and 50 units ml^{-1} penicillin, gassed with 5% CO_2 in air. Cells of either cell line were grown in 75 cm^2 plastic flasks at 37 °C. The medium was changed 3 times a week, and the cells were grown to confluency. For electrophysiological recordings, trypsinized cells were seeded at a density of 200–1000 cells mm^{-2} in plastic Petri dishes containing glass slides and grown for 2–5 days. If indicated, PTX was added to the medium at concentrations of 100 ng ml^{-1} 24 h prior to experiments.

Electrophysiological measurements and data analysis

Glass slides with adherent cells were transferred into a perfusion chamber (volume, 0·2 ml; perfusion rate, 4 ml min^{-1}) which was mounted on an inverted microscope. Whole-cell currents were measured according to Hamill, Marty, Neher, Sakmann & Sigworth (1981) with an L/M-EPC7 patch clamp amplifier (List Electronic) at 36 °C. The resistance of the patch pipettes was 2·5–3·5 MΩ. For infusion experiments, the resistance was 1·8–2·6 MΩ. The series resistance compensation was set to 50–70%. The capacitance of GH_3 cells was 14·0 ± 4·4 pF ($n = 51$; mean ± S.D.), and that of RINm5F cells was 13·6 ± 2 pF ($n = 27$). Two pipette solutions were used at 36 °C. Solution I1 contained (mM): 125 CsCl, 1 $MgCl_2$, 3 MgATP, 10 EGTA, 10 Hepes (pH 7·4). Solution I2 contained (mM): 115 CsCl, 1 $MgCl_2$, 3 MgATP, 20 BAPTA, 10 Hepes (pH 7·4). Intracellular application of GTP did not modify responses, which was in accordance with observations made in an earlier study (Hescheler, Rosenthal, Trautwein & Schultz, 1987). Cells were first superfused with extracellular solution E1 containing (mM): 140 NaCl, 1·8 $CaCl_2$, 1 $MgCl_2$, 5·4 KCl, 10 glucose, 10 Hepes (pH 7·4) at 36 °C. Currents through voltage-operated calcium channels were measured with barium as charge carrier. The solutions used were solution E2 (mM: 125 NaCl, 10 $BaCl_2$, 1 $MgCl_2$, 5·4 CsCl, 10 glucose, 10 Hepes (pH 7·4) at 36 °C) in the case of GH_3 cells or solution E3 (mM: 10 $BaCl_2$, 1 $MgCl_2$, 5·4 CsCl, 10 glucose, 10 Hepes, 125 mM *N*-methyl-D-glucamine (pH 7·4) at 36 °C) for RINm5F cells.

Calcium channel currents were recorded during 20 ms test voltage pulses to 0 mV from a holding potential of −80 mV. The pulses were applied every 2–4 s before, during and shortly after superfusion of a cell with a hormone. The stimulation was not continued between hormonal applications during long-term infusion of Gα subunits in order to avoid excessive run-down of currents. Inhibition of I_{Ca} was calculated as the difference between peak I_{Ca} amplitudes after superfusion of the cells for 8–15 s with a hormone and the mean value of the current amplitudes before the hormone application and after wash-out of the hormone. The current traces, shown in the figures, were not corrected for leak, as this does not contribute to the current measured at 0 mV. For current–voltage relations, a leak current was subtracted from measured peak I_{Ca} amplitudes. The ohmic conductance was calculated from the linear regression of current amplitudes at −80 to −50 mV, assuming 0 pA current at 0 mV. In indicated cases, the background conductance was determined from linear regression of current amplitudes at −60 to +30 mV after addition of 100 μM $CdCl_2$.

Data are given as mean values ± standard deviation (S.D.). Statistical significance was tested with Student's *t* test for paired or unpaired data.

Purification and infusion of G proteins

For isolation of heterotrimeric G proteins and Gα subunits from various tissues and cells, standard techniques were used (Sternweis & Pang, 1990; Codina, Carty, Birnbaumer & Iyengar, 1991). $G\alpha_{i2}$ and $G\alpha_{i3}$ subunits were purified from human thrombocytes, whereas $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{o1}$ and $G\alpha_{o2}$ subunits were from bovine brain. In addition, G_i–G_o mixtures, $G\beta\gamma$ complexes and $G\alpha_{o1}$ subunits were purified from porcine brain. Final resolution of Gα subunits was achieved by Mono Q fast performance liquid chromatography (Nürnberg *et al.* 1994). Purities of Gα isoforms, $G_{i/o}$ mixtures and $G\beta\gamma$ complexes were greater than 95% as determined by silver staining of 10% SDS–polyacrylamide gels. The purified PTX-sensitive Gα isoforms were identified by immunoreactivity to subtype-specific polyclonal antibodies (Spicher *et al.* 1991; Spicher, Nürnberg, Jäger, Rosenthal & Schultz, 1992; Nürnberg *et al.* 1994) and proved to be devoid of contamination by other PTX-sensitive Gα subunits, as assessed by autoradiographic analysis after PTX-mediated ^{32}P-ADP ribosylation; standard protocols were applied for PTX-mediated ADP ribosylation (Nürnberg, 1997). ^{35}S-GTPγS-binding to G proteins was performed as described elsewhere

(Nürnberg *et al.* 1994). The mean value of all measurements was used for obtaining the concentration of a specific G protein preparation.

Purified Gα subunits were stored at −70 °C and diluted immediately before infusion into cells in solution I1 supplemented with Lubrol PX (0·03%; Sigma) and bovine serum and bovine serum albumin (0·3%) to a final concentration of 12 nM. Only those experiments in which I_{Ca} remained reasonably stable over a time period of at least 6 min were analysed.

Immunodetection of G_o isoforms in the membranes of GH_3 and RINm5F cells

For detection of G proteins, discontinuous SDS–PAGE was employed according to Laemmli (1970). Separating gels contained 6 M urea and 9% (w/v) acrylamide. Immunoblotting was performed as described, and filter-bound antibodies were visualized by a colour reaction catalysed by goat-anti-rabbit-IgG coupled to peroxidase (dilution, 1:1000; Sigma) and the enhanced chemiluminescence (ECL) Western-blotting detection system (Amersham) (Spicher *et al.* 1992). ECL-stained blots were exposed to X-ray films for 1–20 min. Antiserum AS 6 (anti-$\alpha_{o,common}$) was raised in rabbits against the peptide (c)NLKEDGISAAKDVK which corresponds to the predicted amino acids 22–35 of either $G\alpha_o$ sequence (Spicher *et al.* 1992). Specificities of antisera AS 6, AS 201 and AS 248 for distinct recombinant α-subunits expressed in *E. coli* were tested as detailed previously (Schmidt *et al.* 1991; Spicher *et al.* 1991, 1992).

Photolabelling and immunoprecipitation of membrane proteins

Photoaffinity labelling of Gα subunits was performed essentially as described by Obukhov *et al.* (1996). Membranes from GH_3 or RINm5F cells (200 mg of protein per assay tube) were incubated at 30 °C in a buffer consisting of (mM): 0·1 EDTA, 10 $MgCl_2$, 30 NaCl, 30 GDP, 50 Hepes (pH 7·4) in the absence or presence of hormones. After 5 min of pre-incubation, samples were incubated for another 3 min with 10–30 nM [α-^{32}P]GTP azidoanilide (5 μCi per tube). The final assay volume was 60 μl. The reaction was stopped by cooling the sample on ice. After centrifugation at 12000 *g* (5 min, 4 °C) the pelleted membranes were resuspended in 60 μl of the above buffer devoid of GDP and supplemented with 2 mM glutathione. Suspended membranes were then irradiated for 10 s at 4 °C with a 254 nm UV lamp (Vilber Lourmat, Torcy, France). Following irradiation, membranes were centrifuged and solubilized in 40 μl of 4% (w/v) SDS for 10 min at room temperature. Thereafter, 280 μl of precipitation buffer (1% (w/v) Nonidet P-40, 1% (w/v) desoxycholate, 150 mM NaCl, 1 mM dithiotreitol, 1 mM EDTA, 0·2 mM phenylmethylsulphonyl fluoride, 10 μg ml^{-1} aprotinin, 10 mM Tris-HCl (pH 7·4)) was added. Solubilized membranes were centifuged (12000 *g*, 5 min, 4 °C) to remove insoluble material, and 10–30 μl of antisera was added to the supernatant. Following incubation under constant rotation (2 h, 4 °C), 60 μl of 12·5% (w/v) protein A-Sepharose beads (Sigma) was added, and samples were incubated overnight (4 °C). Sepharose beads were pelleted (12000 *g*, 5 min) and washed twice with 1 ml of washing buffer A (600 mM NaCl, 50 mM Tris-HCl (pH 7·4), 10 mM EDTA) and once with 1 ml of washing buffer B (300 mM NaCl, 100 mM Tris-HCl (pH 7·4), 10 mM EDTA). Fifty microlitres of SDS–PAGE sample buffer was added to the beads, which were then boiled for 1 min and pelleted. The supernatant was applied to SDS–polyacrylamide gels.

RESULTS

Characterization of the calcium channel currents affected by galanin

In accordance with previous reports (Schmidt *et al.* 1991; Gollasch, Haller, Schultz & Hescheler, 1991), most GH_3 cells (about 75%) and RINm5F cells (more than 50%) exhibited only slowly inactivating, high-threshold I_{Ca}. Currents were completely blocked by a non-selective blocker of high-threshold I_{Ca}, Cd^{2+} (50 μM), in both GH_3 cells (Fig. 1*A*, lower panel) and RINm5F cells (not shown). A rapidly inactivating, low-threshold I_{Ca} component was observed in about 25% of GH_3 and 50% of RINm5F cells, but contributed less than 20% (example visible on Fig. 5*B*) of the peak I_{Ca} at a test potential of 0 mV. In line with a previous report (Homaidan, Sharp & Nowak, 1991), this component of I_{Ca} was not affected by galanin. Na^+ currents in GH_3 cells inactivated over less than 1 ms at 0 mV and did not affect measurements of I_{Ca} (Fig. 1*A*). In preliminary experiments with RINm5F cells, Na^+ currents interfered with measurements of I_{Ca}. Therefore, we used a Na^+-free solution (E3) in all further experiments with these cells.

The dihydropyridine (DHP) blocker of L-type I_{Ca}, (+)-isradipine, inhibited peak I_{Ca} in GH_3 cells by 80 ± 6 and 90 ± 7% at concentrations of 1 and 10 μM, respectively (Fig. 1*B*). At the end of 200 ms voltage pulses, the I_{Ca} amplitude was decreased by 93 ± 2 and by 100% in 1 and 10 μM (+)-isradipine, respectively. After inhibition of I_{Ca} with 10 μM (+)-isradipine, there was no significant change in current when galanin (see Fig. 1*B*), carbachol or somatostatin was applied to the cells. Extracellular application of galanin suppressed I_{Ca} by 20 ± 6% in GH_3 cells (see Fig. 1*A*, upper panel and Table 1). No significant difference was found between rat and porcine galanin, indicating that the galanin receptors involved in I_{Ca} inhibition in these two rat cell lines do not show species specificity, as has been supposed previously (Verchere, Kwok & Brown, 1992).

The RINm5F cell line was reported to possess mainly DHP-sensitive I_{Ca}, with ω-CTX GVIA-sensitive I_{Ca} and low-threshold T-type I_{Ca} as minor components (Schmidt *et al.* 1991; Homaidan *et al.* 1991; Sher *et al.* 1992). Superfusion of the cells with 3 μM ω-CTX GVIA, a potent irreversible inhibitor of N-type I_{Ca} and a reversible weak inhibitor of neuroendocrine L-type I_{Ca} (Williams *et al.* 1992), for 1 min resulted in a weak but significant ($P < 0·01$) inhibition of I_{Ca} by 12 ± 3% (Fig. 1*C* and *D*, trace 4). Extracellular application of galanin alone suppressed I_{Ca} by 33 ± 8% in RINm5F cells (see Fig. 1*C–F*, trace 2 and Table 1). Following ω-CTX GVIA treatment, galanin inhibited I_{Ca} by 20 ± 5% (in the presence of ω-CTX GVIA; Fig. 1*C* and *D*, trace 5). (+)-Isradipine (10 μM) inhibited I_{Ca} by 67 ± 6% (Fig. 1*E* and *F*, trace 3). In the presence of (+)-isradipine, applications of galanin resulted in an additional reduction of I_{Ca} by 8 ± 3% (expressed as a percentage of control I_{Ca};

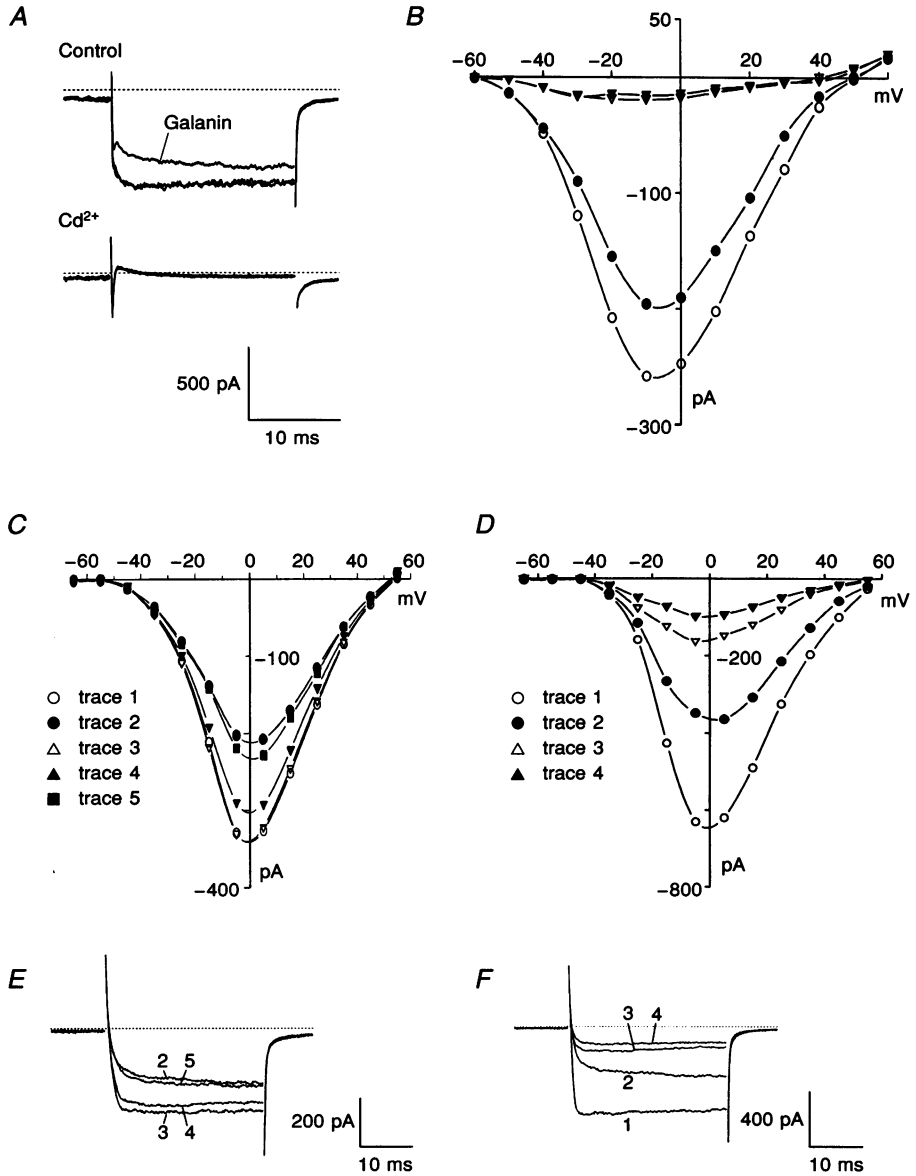

Figure 1. Inhibition of the Ca^{2+} channel current (I_{Ca}) by galanin in GH_3 and RINm5F cells

A, I_{Ca} measured during depolarizing 20 ms voltage pulses from −80 to 0 mV in GH_3 cells. Traces in each panel were recorded before galanin application, under superfusion with galanin and after wash-out of the agonist. All experiments were done during constant perfusion of cells. Upper panel, the effect of galanin (0·5 μM) on I_{Ca} in a cell in control conditions. Lower panel, effect of galanin in the presence of $CdCl_2$ (50 μM). The dotted lines on this and all other figures denote the zero current. *B*, current–voltage (*I–V*) relations of I_{Ca} in GH_3 cells obtained from peak current amplitudes during voltage pulses (30 ms, 0·5 Hz) from a holding-potential of −80 mV. ○ (trace 1), *I–V* relations under control condition without drugs (solutions I1 and E2); ● (trace 2), I_{Ca} after application of galanin (0·5 μM); △ (trace 3), I_{Ca} after application of (+)-isradipine (10 μM); ▲ (trace 4), I_{Ca} after subsequent application of a mixture of galanin and (+)-isradipine. I_{Ca} amplitudes were corrected for leak determined after addition of $CdCl_2$ (100 μM). *C*, the *I–V* relations of I_{Ca} in RINm5F cells during voltage pulses (30 ms, 0·5 Hz) from a holding-potential of −80 mV. ○ (trace 1), I_{Ca} under control conditions; ● (trace 2), I_{Ca} after application of galanin (0·5 μM); △ (trace 3), I_{Ca} after washing of galanin from the bath; ▲ (trace 4), I_{Ca} after incubation with ω-CTX GVIA (3 μM) for 1 min; ■ (trace 5), I_{Ca} after subsequent application of galanin together with ω-CTX GVIA. This experiment was performed at 30 °C in order to decrease the rate of I_{Ca} run-down. *D*, the *I–V* relations of I_{Ca} in RINm5F cells. ○ (trace 1), I_{Ca} under control conditions; ● (trace 2), I_{Ca} after application of galanin (0·5 μM); △ (trace 3), I_{Ca} after addition of (+)-isradipine (10 μM); ▲ (trace 4), I_{Ca} after and subsequent application of a mixture of galanin and (+)-isradipine. *E*, representative I_{Ca} traces at a test potential of −5 mV (numbers correspond to the experimental conditions outlined in panel *C*). *F*, representative I_{Ca} traces at a test potential of −5 mV are shown (numbers correspond to the experimental conditions outlined in panel *D*).

Fig. 1*E* and *F*, trace 4). The combination of 10 μM (+)-isradipine and 3 μM ω-CTX GVIA inhibited I_{Ca} by 70 ± 8%, which is not significantly different from the effect of (+)-isradipine alone. In the presence of these two blockers, galanin produced a marginal additional I_{Ca} inhibition of 6 ± 2% (expressed as a percentage of control I_{Ca}). ω-Aga IVA (0·3 μM), a blocker of P-type I_{Ca} and neuroedocrine L-type I_{Ca} (Aosaki & Kasai, 1989), inhibited I_{Ca} by 14 ± 5% ($P < 0·05$; not shown), which is comparable to the ω-CTX GVIA-induced inhibition. ω-CTX MVIIC (2·5 μM), a blocker of Q- and N-types I_{Ca} (Hillyard *et al.* 1992), did not affect either I_{Ca} or galanin-induced inhibition of I_{Ca} and did not enhance the effect of ω-Aga IVA.

Galanin-induced inhibition of I_{Ca} mediated by PTX-sensitive G proteins

The inhibition of I_{Ca} by galanin appeared soon after application of the peptide and reached its maximum within 3–8 s, the time required for solution exchange in the perfusion chamber in both cell lines. The inhibition was completely reversible after wash-out and could be repeated with subsequent galanin applications. In GH_3 cells, the degree of the inhibition during repeated applications of galanin was unchanged when BAPTA (20 mM in internal solution I2) was used in the pipette solution instead of EGTA (10 mM in solution I1), suggesting that inhibition of I_{Ca} by galanin is associated with more than just Ca^{2+} release from intracellular stores.

The stable GDP analogue guanosine 5′-*O*-[2-thio]diphosphate (GDPβS) stabilizes G proteins in their inactive form. When GDPβS (500 μM) was added to the pipette solution, galanin induced an initial inhibition of I_{Ca} in both cell lines (see Table 1). However, when GDPβS was allowed to diffuse into the cells for more than 4 min, galanin no longer inhibited I_{Ca} (Fig. 2*A* and Table 1). Likewise, pretreatment of cells with pertussis toxin (PTX) significantly ($P < 0·001$) suppressed I_{Ca} inhibition by galanin in both cell lines (see Table 1).

Galanin not only inhibited the peak amplitude of I_{Ca} but also slowed activation of I_{Ca} in RINm5F cells (see Fig. 1*F*, trace 2, and Fig. 2*B*, trace 4). Similar effects on the kinetics of activation have been reported for many other hormones and transmitters (Hille, 1994). They are attributed to a voltage-dependent relief from inhibition (Tsunoo, Yoshii & Narahashi, 1986). In line with this notion, galanin-induced

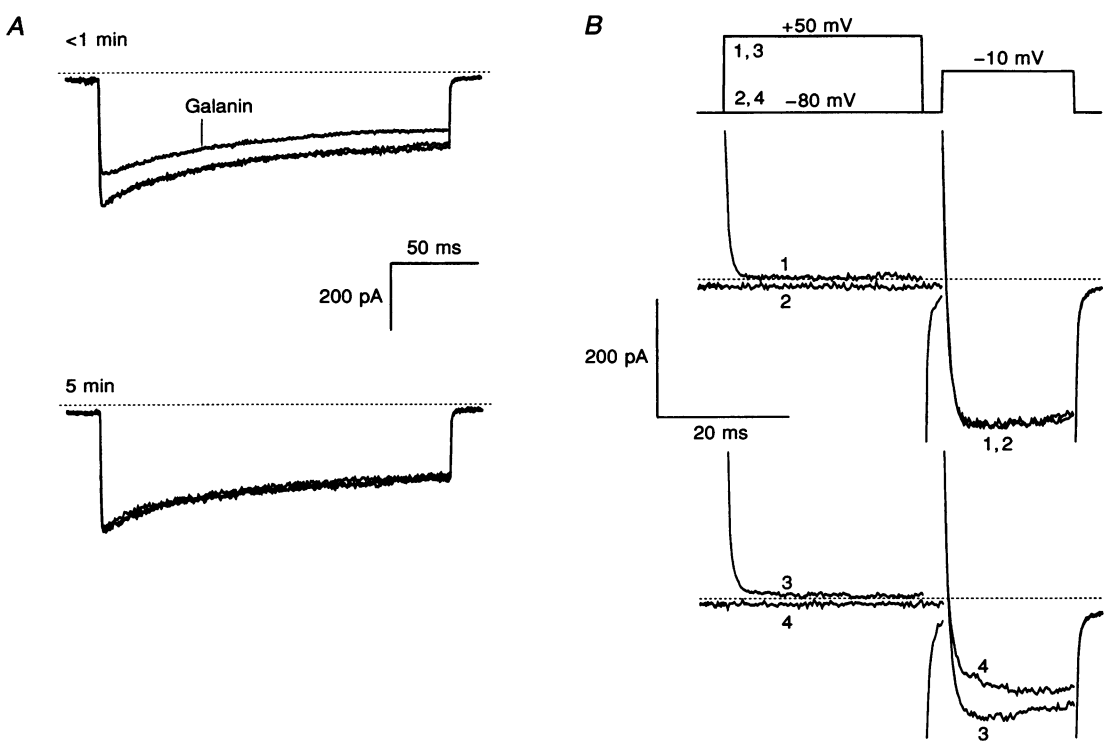

Figure 2. Effect of GDPβS and depolarizing prepulses on galanin-induced inhibition of I_{Ca}

A, I_{Ca} recorded in a GH_3 cell (test pulses from −80 to 0 mV). Traces in each panel record I_{Ca} before (control), during application of galanin and after wash-out. Upper panel, effect of galanin on I_{Ca} during the first minute of GDPβS-infusion (500 μM in solution I1) into the cell through the patch pipette. Lower panel, effect of galanin (0·5 μM) on I_{Ca} in the cell after 5 min of GDPβS infusion. *B*, consecutive traces of I_{Ca} recorded in RINm5F cell using standard (traces 2 and 4) and double-pulse protocols (traces 1 and 3) (see upper panel for pulse protocol scheme). The double-pulse protocol included a depolarizing 30 ms voltage step from −80 to +50 mV preceding the 20 ms test pulse to −10 mV. Middle panel, traces (1 and 2) in the absence of galanin. Bottom panel, I_{Ca} after galanin application.

Table 1. Effects of pertussis toxin, GDPβS and infused G protein α subunits on the hormonal inhibition of I_{Ca}

Treatment	Infusion time (min)	GH_3 cells			RINm5F cells
		Galanin (%)	Carbachol (%)	Somatostatin (%)	Galanin (%)
Control		20 ± 6 ($n = 9$, $m = 15$)	20 ± 4 ($n = 4$, $m = 6$)	19 ± 5 ($n = 6$, $m = 8$)	33 ± 8 ($n = 24$, $m = 45$)
GDPβS	< 1	20 ± 4 ($n = m = 3$)	—	—	39 ± 5 ($n = m = 2$)
	> 4	4 ± 3 ($n = 3$, $m = 6$)	—	—	5 ± 2 ($n = 2$, $m = 4$)
PTX		6 ± 3 ($n = 33$, $m = 59$)	6 ± 3 ($n = 4$, $m = 5$)	6 ± 3 ($n = 6$, $m = 7$)	7 ± 6 ($n = 47$, $m = 76$)
$G\alpha_{o1}$	< 3	8 ± 6 ($n = m = 10$)	5 ± 3 ($n = m = 3$)	4 ± 3 ($n = m = 5$)	6 ± 6 ($n = m = 19$)
	> 5	17 ± 5 ($n = 10$, $m = 19$)**	18 ± 4 ($n = 3$, $m = 8$)*	6 ± 5 ($n = m = 4$)	14 ± 8 ($n = 19$, $m = 45$)**
$G\alpha_{o2}$	< 3	6 ± 4 ($n = m = 8$)	3 ± 2 ($n = m = 3$)	6 ± 6 ($n = m = 6$)	7 ± 4 ($n = m = 6$)
	> 5	5 ± 2 ($n = 5$, $m = 10$)	4 ± 2 ($n = 3$, $m = 7$)	17 ± 5 ($n = m = 5$)*	7 ± 5 ($n = 5$, $m = 17$)
$G\alpha_{o1}$, $G\alpha_{o2}$	< 3	5 ± 4 ($n = m = 4$)	—	—	6 ± 5 ($n = m = 9$)
	> 5	15 ± 3 ($n = 4$, $m = 9$)**	—	—	13 ± 4 ($n = 9$, $m = 18$)**
$G\alpha_{i1}$,$G\alpha_{i2}$,$G\alpha_{i3}$	< 3	6 ± 1 ($n = m = 2$)	—	—	3 ± 2 ($n = m = 4$)
	> 5	5 ± 3 ($n = m = 4$)	3 ± 2 ($n = m = 3$)	6 ± 6 ($n = m = 3$)	4 ± 3 ($n = 4$, $m = 9$)

Inhibition of I_{Ca} by galanin (0·5 μM), carbachol (10 μM) and somatostatin (1 μM) in GH_3 cells and by galanin (0·5 μM) only in RINm5F cells is shown after infusion of GDPβS (0·5 mM), after pretreatment with PTX or after infusion of Gα isoforms for different times. Percentage inhibition values are given as means ± S.D. from different cells (calculated from number of cells, n; total number of hormone applications (m) also given). Effects that developed over time following Gα infusion and which are significantly different from the effects in PTX-treated cells are indicated by asterisks: ** $P < 0·001$; * $P < 0·05$.

inhibition of I_{Ca} in RINm5F cells was partially relieved by large depolarizing prepulses (30 ms, +50 mV; see Fig. 2*B*, traces 3 and 4). As a parameter reflecting the activation kinetics of I_{Ca}, we measured the time-to-peak I_{Ca} during a voltage pulse to −10 mV in RINm5F cells. In the absence of galanin, this was 5·5 ± 1·2 ms without prepulses and 5·6 ± 1·2 ms following prepulses. In the presence of galanin, time-to-peak I_{Ca} increased to 17 ± 2·8 ms without prepulses. Prepulses reduced this time to 7·6 ± 1·7 ms ($P < 0·01$).

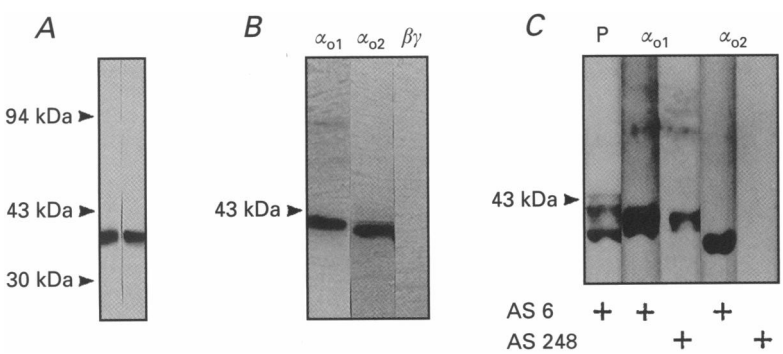

Figure 3. Identification of purified $G\alpha_o$ subtypes

A, silver stain of SDS–polyacrylamide gels. Purified $G\alpha_{o1}$ and $G\alpha_{o2}$ (500 ng each), respectively, were loaded on the gels (10% acrylamide, 9 cm length, 4 mm width of slots), separated and silver stained. Molecular masses of marker proteins are indicated. *B*, PTX-mediated ADP ribosylation of purified $G\alpha_{o1}$ and $G\alpha_{o2}$. Purified proteins (500 ng each) were treated with PTX in the presence of [^{32}P]NAD and purified brain $G\beta\gamma$ subunits. For control, autoradiograph of the incubation mixture containing only PTX, [^{32}P]NAD and $G\beta\gamma$ dimers is shown on the right lane. Proteins were subsequently resolved on gels (6 M urea, 9% acrylamide, 13 cm length, 4 mm width of slots), blotted and nitrocellulose filters exposed to X-ray films. *C*, immunostain of filter-bound antibodies. Acetone-precipitated $G\alpha_{o1}$, $G\alpha_{o2}$ and a mixture of both proteins (P) were loaded on gels (6 M urea, 9% acrylamide, 13 cm length, 4 mm width of slots). After blotting, nitrocellulose-filters were incubated with antibodies AS 6 ($\alpha_{o,common}$ antiserum, diluted 1:300) and AS 248 (α_{o1}-antibodies, affinity purified). The ECL system was used for detection of filter-bound antibodies. Procedures were carried out as described in Methods. Molecular markers are indicated.

Restitution of receptor-mediated I_{Ca} inhibition by infusion of purified Gα isoforms

To identify the G proteins involved in the inhibition of I_{Ca} by galanin, we infused different purified PTX-sensitive Gα isoforms into PTX-treated GH_3 and RINm5F cells (Fig. 3). In either cell line, galanin-induced inhibition of voltage-activated calcium channels was restored by infusion of $G\alpha_{o1}$ subunits but not by infusion of $G\alpha_{o2}$ subunits (12 nM in the pipette solution; Figs 4 and 5 and Table 1). Furthermore, in GH_3 cells, infusion of $G\alpha_{o1}$ subunits restored the I_{Ca} response to carbachol but not to somatostatin (see Fig. 4*A* and *B* and Table 1). Conversely, infusion of $G\alpha_{o2}$ into GH_3 cells restored the I_{Ca} response to somatostatin but not to galanin or carbachol (see Fig. 4*C* and Table 1). Infusion of a mixture of $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$ subunits (12 nM of each subunit) did not restore I_{Ca} responses to galanin in either GH_3 (see Table 1) or RINm5F cells (see Fig. 5 and Table 1). However, in a different study, purified $G\alpha_i$ proteins of the same batch were able to stimulate phosphatidylinositide-3 kinase γ activity in a concentration-dependent manner (Stoyanov *et al.* 1995). Infusion of a mixture of $G\alpha_{o1}$ and $G\alpha_{o2}$ subunits (12 nM each) or of $G\alpha_{o1}$ subunits alone into cells of either line restored galanin-induced inhibition of I_{Ca} to the same extent.

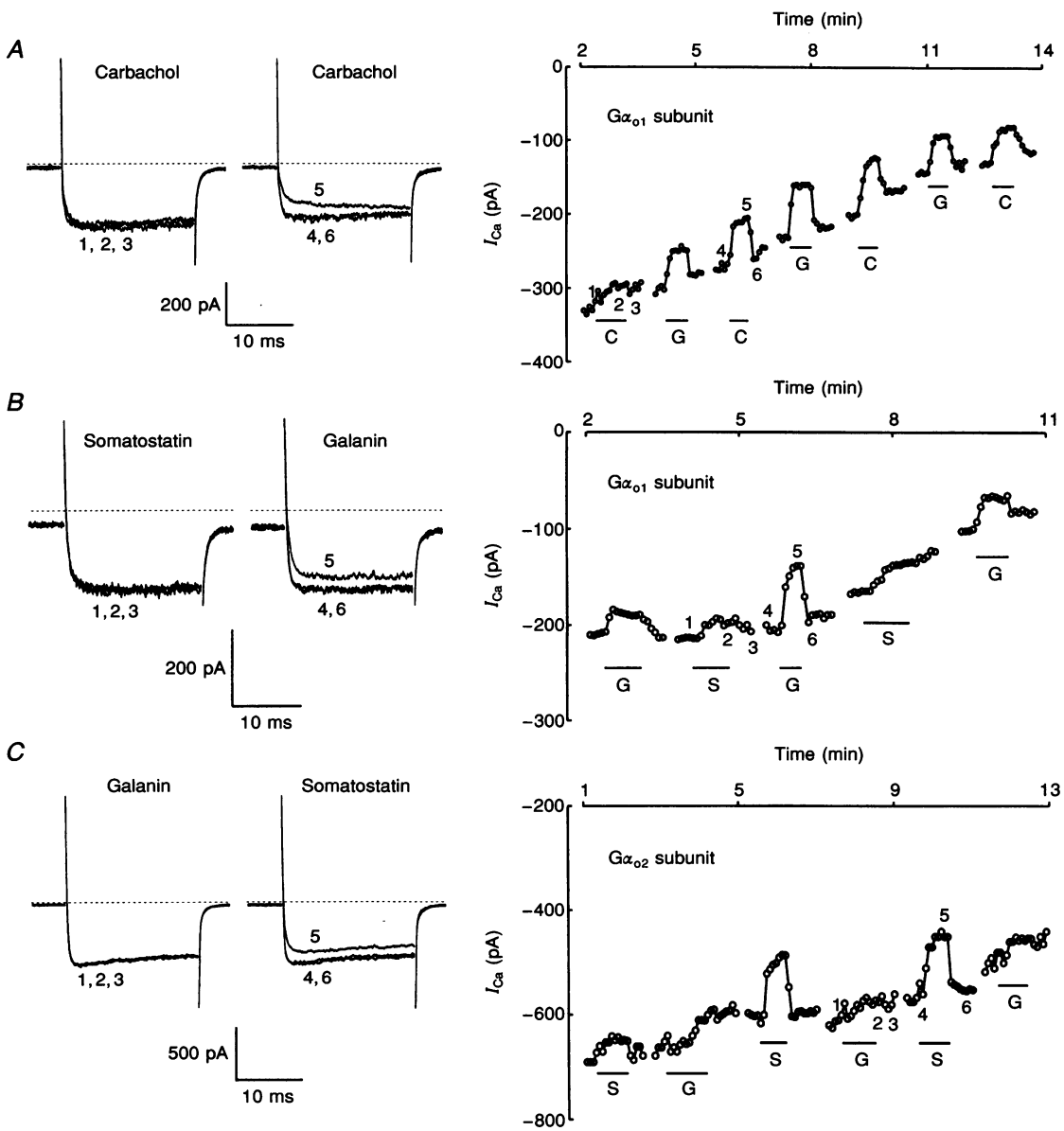

Figure 4. Reconstitution of hormonal inhibition of I_{Ca} in PTX-pretreated GH_3 cells infused with GDP-bound inactive $G\alpha_{o1}$ and $G\alpha_{o2}$ subunits (12 nM)

Left panels show I_{Ca} traces recorded at time points 1–6 indicated on the right panels. *A*, effects of galanin (G) and carbachol (C) on I_{Ca} during infusion of $G\alpha_{o1}$. *B*, effects of galanin and somatostatin (S) during infusion of $G\alpha_{o1}$. *C*, effect of somatostatin and galanin during infusion of $G\alpha_{o2}$.

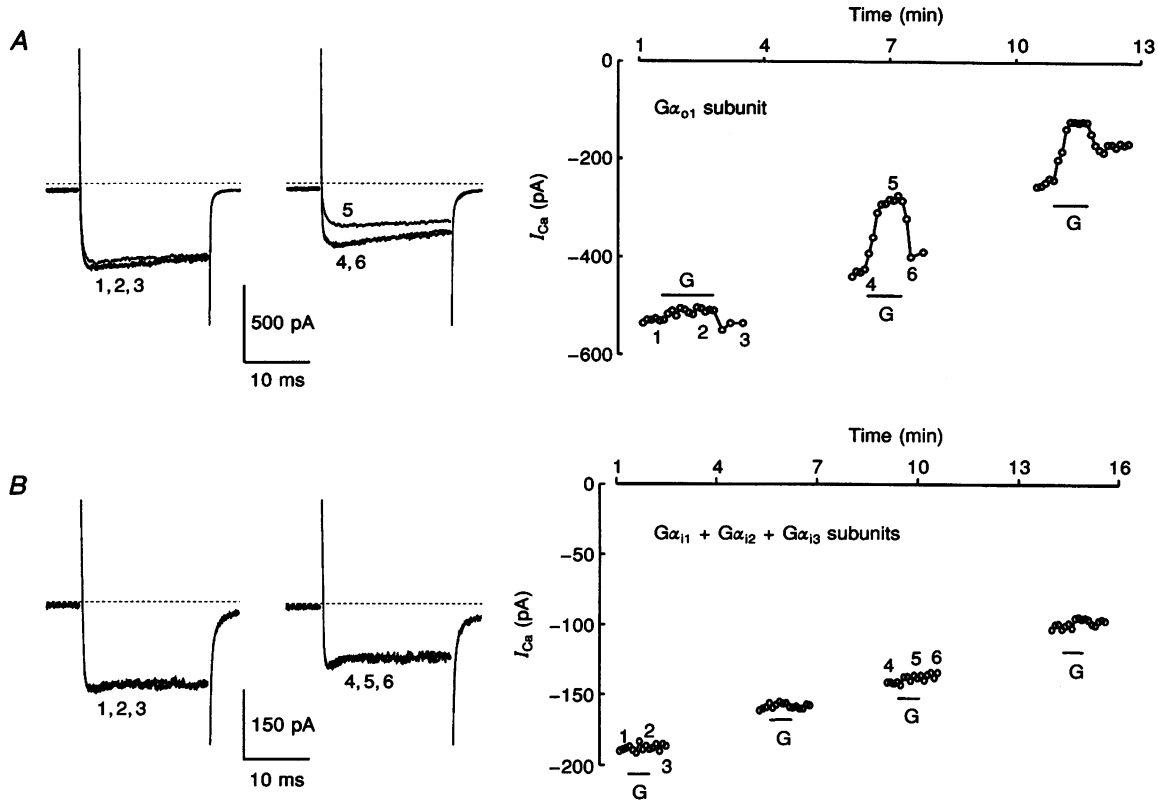

Figure 5. Reconstitution of hormonal inhibition of I_{Ca} in PTX-pretreated RINm5F cells infused with GDP-bound inactive $G\alpha_{o1}$ and $G\alpha_{i}$ subunits (12 nM)

Left panels show I_{Ca} traces recorded at time points 1–6 indicated on the right panels. *A*, effects of galanin (G) on I_{Ca} during infusion of $G\alpha_{o1}$. *B*, effects of galanin during infusion of $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$.

In experiments with GH_3 cells, inhibition of I_{Ca} by all three hormones was almost completely restored by infusion of appropriate $G\alpha_o$ isoforms for 16 ± 8 min ($n = 22$; see Table 1). Slightly different results were obtained with RINm5F cells. In PTX-treated RINm5F cells restoration of I_{Ca} inhibition was still incomplete after infusion of $G\alpha_{o1}$ subunits for 13 ± 5 min ($n = 19$; Fig. 5*A*). In control cells, the first galanin application following achievement of a whole-cell clamp resulted in a mean I_{Ca} inhibition of $33 \pm 8\%$, but subsequent applications were less effective. We noted that our preliminary data indicated that the diminishing response correlated with the time of whole-cell recording rather than the number of galanin applications. In control experiments, mean I_{Ca} inhibition by galanin was only $26{\cdot}2 \pm 1{\cdot}7\%$ after 13 min, a value closer to the mean inhibition observed after infusion of purified $G\alpha$ (see Table 1).

Hormonal stimulation of G_o proteins in the membranes of GH_3 and RINm5F cells

Both cell lines express various isoforms of PTX-sensitive G_i and G_o proteins. To obtain a rough estimate of the quantitative expression of G_o isoforms we examined the intensity of immunostaining by antibodies specific to the different G protein subtypes. Immunoblotting of membrane proteins derived from GH_3 cells exhibited two $\alpha_{o,common}$ immunoreactive bands after staining with AS 6 ($\alpha_{o,common}$ anti-

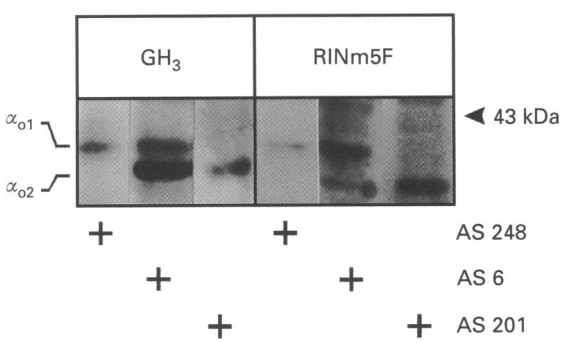

Figure 6. Presence of $G\alpha_o$ subtypes in the membranes of GH_3 and RINm5F cells

Membranes (100 μg of protein) of GH_3 and RINm5F cells were acetone-precipitated and loaded on SDS–polyacrylamide gels (6 M urea, 9% acrylamide, 13 cm length, 4 mm width of slots). After blotting, nitrocellulose filters were incubated with antiserum AS 6 ($\alpha_{o,common}$ antiserum, diluted 1:300), AS 248 (α_{o1} antibodies, affinity purified) and AS 201 (α_{o2} antiserum, 1:150). The ECL system was used for detection of filter-bound antibodies. Molecular marker is indicated.

serum; Fig. 6). Of the two immunoreactive bands, only the upper one was detected by a $G\alpha_{o1}$-specific antibody (AS 248; see Fig. 6), whereas the lower band was sensitive to an antiserum specific for rodent $G\alpha_{o2}$ (AS 201; see Fig. 6). Although the anti-$\alpha_{o,common}$ antiserum (AS 6) recognizes an identical amino acid sequence region near the *N*-terminus of both $G\alpha_o$ subtypes (Spicher *et al.* 1992), the upper band was less intensively stained than the lower in membranes from GH_3 cells whereas the lower band was the lighter in membranes from RINm5F cells.

Next, we examined which $G\alpha_o$ isoforms are activated by receptors inhibiting voltage-operated calcium channels in GH_3 and RINm5F cells. Membranes were prepared from GH_3 and RINm5F cells and incubated with receptor agonists in the presence of the photoreactive GTP analogue [α-^{32}P]GTP azidoanilide (Fig. 7). As illustrated in Fig. 7 (left panel), all receptor agonists used, i.e. galanin, as well as carbachol and somatostatin, were applied to membranes of GH_3 cells and increased the amount of radioactivity visible in immunopecipitates from $G\alpha_o$-selective antisera compared with unstimulated controls. In particular, results with the $G\alpha_{o2}$-selective antiserum AS 201 implied that stimulation of receptors for galanin, M4 and somatostatin activated G_{o2} proteins. Unfortunately, the $G\alpha_{o1}$-specific antiserum AS 248 does not precipitate sufficient quantities of protein. Therefore, we applied antiserum AS 6 which recognizes both $G\alpha_o$ isoforms. Use of this antiserum in combination with high resolution SDS–PAGE allowed discrimination of protein bands belonging to each $G\alpha_o$ isoform. As shown in Fig. 7, immunoprecipitates from antiserum AS 6 were separated by SDS–PAGE into two bands, the faster migrating of which had an apparent molecular weight similar to that of $G\alpha_{o2}$ precipitated by AS 201. This faster-migrating band exhibited increased radioactivity after agonist stimulation of membranes, confirming that it represents receptor-induced activation of $G\alpha_{o2}$. Based on the electrophoretic mobility of $G\alpha_o$ isoforms visible in immunoblot experiments (see Fig. 6), it is feasible to conclude that the slower-migrating band seen in autoradiograms of SDS–PAGE-separated AS 6 immunoprecipitates represented $G\alpha_{o1}$ subunits (see Fig. 7). In GH_3 cell membranes, all agonists also increased the radioactivity incorporated into this $G\alpha_o$ isoform. In conclusion, in GH_3 cell membranes, all three agonists induced activation of both $G\alpha_{o1}$ and $G\alpha_{o2}$ subunits.

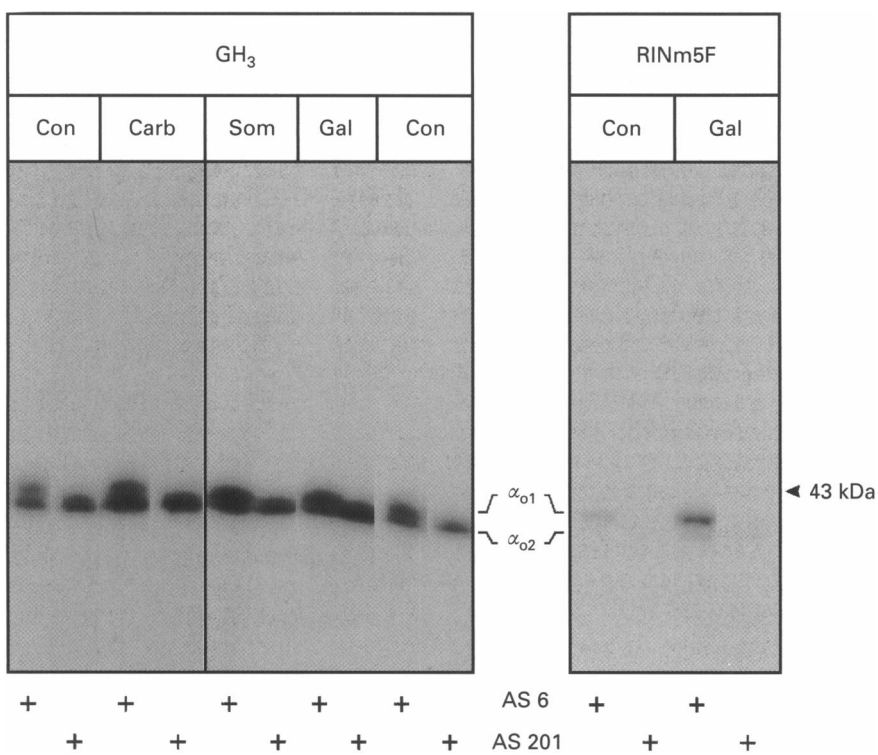

Figure 7. Receptor-mediated [α-^{32}P]GTP azidoanilide photolabelling of $G\alpha_o$ subtypes in GH_3 and RINm5F cell membranes

For photolabelling and immunoprecipitation of $G\alpha$ proteins incubation of membranes with [α-^{32}P]GTP azidoanilide was performed in the absence (Con) or presence of agonists, i.e. carbachol (Carb; 30 μM), somatostatin (Som; 1 μM), and galanin (Gal; 1 μM), as described in Methods. AS 6 precipitated $G\alpha_{o1}$ (upper bands) and $G\alpha_{o2}$ (lower bands), whereas AS 201 was specific for $G\alpha_{o2}$. Apparent molecular mass of a marker protein is indicated on the left. Autoradiograms shown are representative of three independent experiments with similar results.

In RINm5F cell membranes, $G\alpha_{o1}$ is the predominant $G\alpha_o$ isoform (see Fig. 6). Incubation of RINm5F cell membranes with galanin clearly activated $G\alpha_{o1}$ (see Fig. 7, right panel). Experiments with AS 6 showed a faster-migrating band (representing $G\alpha_{o2}$) after incubation of cell membranes with galanin. Several experiments using AS 201 (anti-α_{o2}) showed faint bands after stimulation with galanin. Therefore, we conclude that in RINm5F cells galanin obviously activated $G\alpha_{o1}$ subunits and probably activated $G\alpha_{o2}$ subunits also.

DISCUSSION

In this study we examined signalling pathways from hormonal cell surface receptors to voltage-gated calcium channels. First, the type of calcium channel affected by galanin in two neuroendocrine cell lines (GH_3 and RINm5F cells), and by somatostatin and carbachol in GH_3 cells only, was determined. GH_3 cells were reported to express α_{1A}, α_{1C} and α_{1D} calcium channel-forming subunits (Lievano, Bolden & Horn, 1994). In this study, we used a GH_3 clone which expresses only DHP-sensitive L-type calcium channels (demonstrated here and in a previous study by Gollasch *et al.* 1991). RINm5F cells exhibited at least three types of calcium channel contributing to high-threshold I_{Ca}: an ω-CTX GVIA-resistant, DHP-sensitive type, an ω-CTX GVIA-sensitive type, and an ω-CTX GVIA- and DHP-resistant type. The major part of I_{Ca} inhibition by galanin in RINm5F cells was due to the block of DHP-sensitive L-type calcium channels. Results showing the effects of (+)-isradipine and ω-CTX GVIA on I_{Ca} in RINm5F cells suggest the presence of neuroendocrine α_{1D}- and α_{1C}-like subunits of L-type calcium channels, rather than neuronal N-type channels (see Fig. 1). ω-CTX GVIA, a strong blocker of N-type channels and a weak blocker of neuroendocrine L-type channels (Williams *et al.* 1992), did not enhance the block of I_{Ca} by DHP. Inhibition of I_{Ca} by ω-Aga IVA may indicate expression of P-type channels, but since neuroendocrine L-type channels are also sensitive to this toxin (Aosaki & Kasai, 1989), the effects of ω-Aga IVA were presumably caused by block of neuroendocrine L-type I_{Ca}.

The results indicate that the L-type calcium channels detected in insulinoma RINm5F cells are sensitive to galanin. Most probably, of the various L-type calcium channels present, the α_{1D} isoforms were affected by galanin, since ω-CTX GVIA decreased the portion of currents inhibited by the hormone. In accordance with our results, α_{1C} and α_{1D} subunits have previously been detected in endocrine pancreas (Seino *et al.* 1992; Pollo, Lovallo, Biancardi, Sher, Socci & Carbone, 1993; Ihara *et al.* 1995) and have also been found in a hamster insulinoma (HIT) cell line (Boyd *et al.* 1991; Yaney *et al.* 1992). In RINm5F cells, galanin-induced inhibition of I_{Ca} was similar to that induced by many other hormones. Calcium channel inhibition developed fast and I_{Ca} recovered quickly after wash-out. Like noradrenaline or adrenaline in RINm5F cells (Aicardi, Pollo, Sher & Carbone, 1991), and many other transmitters acting via PTX-sensitive G proteins in other models (Bean, 1989; Swandulla, Carbone & Lux, 1991; Hille, 1994), galanin slowed down the activation of I_{Ca} in a voltage-dependent manner and the effect could be relieved by preconditioning depolarization.

The present study identified the G proteins involved in galanin-induced inhibition of I_{Ca} in RINm5F and GH_3 cells by infusion of purified inactive GDP-bound $G\alpha$ subunits following PTX treatment of the cells. Restoration of galanin-induced inhibition of I_{Ca} in RINm5F cells, and of inhibition of I_{Ca} by galanin, carbachol and somatostatin in GH_3 cells, revealed that specific $G\alpha_o$ isoforms are involved in the signal transduction from receptors to L-type Ca^{2+} channels. The existence of specific $G\alpha$ isoforms mediating receptor-induced I_{Ca} inhibition was previously recognized using another experimental approach, i.e. functional knock-out by antisense-oligonucleotide injection (Kleuss *et al.* 1993; Campbell, Berrow & Dolphin, 1993; Kalkbrenner *et al.* 1995). Here we show for the first time that a different technique, i.e. reconstitution of the missing signal transduction element, has identified the same $G\alpha$ isoforms. Infusion of $G\alpha_o$ isoforms into PTX-treated GH_3 and RINm5F cells restored the hormone-induced inhibition of L-type I_{Ca} in a remarkably specific fashion in whole cells. These experiments exclude G_i proteins as mediators of I_{Ca} inhibition and show that the $G\alpha_o$ isoform which restores receptor-induced inhibition of I_{Ca} is agonist specific. Galanin and muscarinic receptors signalled through activation of $G\alpha_{o1}$ subunits, whereas somatostatin receptors inhibited I_{Ca} through activation of G_{o2} subunits. The specificity of G protein coupling was confined to the receptor level, since either G_o splice variant inhibited voltage-operated calcium channels. The observed specificity in receptor–G protein coupling is independent of the two cell lines studied, which exhibit quantitative differences in G_o isoform expression (see Fig. 6).

Functional knock-out of endogenous G proteins was accomplished by PTX-mediated ADP ribosylation of $G\alpha$ subunits (reviewed in Nürnberg, 1997). This manipulation prevents activation of the G protein by receptors but does not alter the quantitative composition of the signal transduction elements in the cell membrane. However, the PTX-treated ADP-ribosylated $G\alpha$ subunit still undergoes receptor-independent activation–inactivation cycles, because there is a low basal level of GTP exchange for GDP on the $G\alpha$ subunit. Inactivated GDP-bound $G\alpha$ subunits infused into the cell are not able either to associate with the membrane (Sternweis, 1986; Iniguez-Lluhi, Kleuss & Gilman, 1993) or to modulate effectors (since they are in their inactive state). However, when the membrane-associated PTX-modified $G\alpha$ changes in its active conformation by binding to GTP, it simultanously dissociates from its membrane-associated $G\beta\gamma$ complex (reviewed in Nürnberg & Ahnert-Hilger, 1996). Meanwhile, it is likely that the $G\beta\gamma$ complex of G_o remains associated with the receptor (Kelleher & Johnson, 1988; Fawzi, Fay, Murphy, Tamir, Erdos & Northrup, 1991; Phillips & Cerione, 1992). When

the receptor-uncoupled, PTX-modified endogenous Gα subunit reassociates with endogenous membrane-attached Gβγ it competes with the infused inactive GDP-bound Gα for binding to Gβγ. This results in an increasing pool of intact heterotrimeric G proteins associated with receptors, thereby allowing receptor-induced activation and release of G protein subunits to modulate cellular effectors. In conclusion, endogenous Gβγ may function as a specific membrane acceptor site for infused Gα.

This study was initiated to identify the G protein responsible for receptor-mediated I_{Ca} inhibition, from which the subunit is released to interact with calcium channels. Although our results do not imply that the Gα subunit interacts with the effector, various studies with purified proteins or membrane preparations have implied that promiscuity occurs at the level of Gα–Gβγ interaction, as well as during receptor–G protein coupling (reviewed in Nürnberg *et al.* 1995). Those results may be in contrast to our findings obtained by infusion of Gα isoforms into PTX-treated cells. In whole cells, only $G\alpha_{o1}$, but not $G\alpha_{o2}$ or $G\alpha_{i}$, isoforms inhibited I_{Ca}. In membrane preparations of either cell line, receptors for galanin, as well as for all other agonists studied, coupled to G_o and G_i proteins (see Fig. 7; Schmidt *et al.* 1991; Offermanns *et al.* 1991; Law *et al.* 1993; Hadcock *et al.* 1994; Bedecs, Berthold & Bartfai, 1995). The endogenous Gβγ complex associating with the $G\alpha_{o1}$ subunit has previously been shown to consist of $G\beta_2\gamma_2$ or $G\beta_3\gamma_4$ but not other Gβγ complexes. In addition, the same $G\beta_2\gamma_2$ complex was also found to couple specifically to the $G\alpha_{13}$ subunit for adenosine A_3 receptor-mediated phospholipase-Cβ activation in RBL-2H3-hm1 cells (Kalkbrenner *et al.* 1996). Previously the $G\alpha_{i}$ subunit was found to couple to galanin receptors (Schmidt *et al.* 1991). Therefore, it was reasonable to assume that infused $G\alpha_{i}$ would have associated with membrane-bound $G\beta_2\gamma_2$ complexes and would therefore be expected to restore receptor-mediated I_{Ca} inhibition. This is even more likely, since it has recently been suggested that Gβγ complexes modulate α_{1A} and α_{1B} calcium channel-forming subunits (Herlitze, Garcia, Mackie, Hille, Scheuer & Catterall, 1996; Ikeda, 1996). In our experiments, the $G\alpha_{i}$ subunit failed to restore receptor-mediated calcium channel inhibition. This observation suggested that Gβγ complexes alone do not control G protein specificity in receptor-mediated I_{Ca} inhibition. Even a combination of a particular receptor and a distinct Gαβγ complex cannot account for the observed specificity in receptor–G protein effector coupling. Hence, our study implies that additional factors are involved in G protein specificity of signalling pathways. In searching for the factors, one has to keep in mind that the high specificity observed is confined to whole cells. In other words, there must be a subtle compartmentation within the cell which is lost when the cells are disrupted and fractionated. Previous studies recognized membrane domains such as caveolae (Chang *et al.* 1994; reviewed in Neubig, 1994). Morphological studies are required to confirm the observed functional compartmentation of signal transduction components.

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