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Transfected Muscarinic Acetylcholine Receptors Selectively Couple to G_i-Type G Proteins and $G_{q/11}$

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SUMMARY

Regulation of effector functions by muscarinic acetylcholine receptor subtypes is mediated by pertussis toxin-sensitive and -insensitive G proteins. In membranes from human embryonic kidney 293 cells transfected with m1, m2, and m3 muscarinic acetylcholine receptors, we detected the pertussis toxin-sensitive G proteins G_{i1}, G_{i2}, and G_{i3} and the pertussis toxin-insensitive G proteins $G_{q/11}$ and G_s. Subtype-specific immunoprecipitation of G protein α subunits photolabeled with [α-^{32}P]GTP azidoanilide, in the absence and presence of carbachol, revealed the selective coupling of activated muscarinic receptors to G protein subtypes. $G_{q/11}$ was activated via m1 and m3 receptors and G_{i2} was activated via m2 receptors. All three receptor subtypes mediated the activation of G_{i1} and G_{i3}. Effective activation of G_{i1} and G_{i3} via m1 and m3 receptors occurred only at high carbachol concentrations (EC_{50} about 10–20 μM), whereas carbachol with higher potency (EC_{50} about 1 μM) induced activation of all G_i subtypes via m2 receptors. Thus, coupling of muscarinic receptors and G protein subtypes was principally selective; however, activation of distinct G protein subtypes by different muscarinic receptors occurred with different efficacies.

The mAchRs belong to the family of seven-transmembrane domain receptors that interact with G proteins to evoke intracellular responses. The group of mAchRs consists of five subtypes (m1–m5), which are encoded by different genes (1–4). Individual mAchRs are functionally different when expressed in cultured cells. Whereas the m1, m3, and m5 subtypes have been shown to activate pathways leading to potent stimulation of PLC as well as of phospholipase A_2 and PLD (5–7), the m2 and m4 subtypes mediate inhibition of adenylyl cyclase (5). At comparably high agonist concentrations, the activated m2 and m4 subtypes can also induce the stimulation of PLC.

The G proteins involved in the signaling pathways activated by the m1, m3, and m5 subtypes and by the m2 and m4 subtypes have been partially characterized by the effect of PTX. Effects mediated by m1, m3, and m5 subtypes are mainly resistant to the action of PTX (8, 9), whereas PTX blocks the effects induced via activation of m2 and m4 subtypes (8, 10). Consistent with that, *in vitro* reconstitution experiments showed that activation of the β1 isoform of PLC by m1 receptors can be mediated by the PTX-insensitive G proteins $G_{q/11}$[2] (11) and that the activated m2 receptor, in contrast to the m1 subtype, efficiently couples to the PTX-sensitive G_i- and G_o-type G proteins (12). Finally, transfected m2 mAchRs, but not the m3 subtype, couple to G_{i2} and G_{i3} in CHO cells (13).

To identify the PTX-sensitive and -insensitive G proteins activated by mAchR subtypes, we studied the interaction of receptors with individual G proteins in membranes of HEK cells that had been stably transfected with the human m1, m2, and m3 mAchRs. These cells have previously been used to elucidate the functional characteristics of the mAchR subtypes (5, 7). Using a combination of receptor-stimulated photolabeling of G protein α subunits and subsequent immunoprecipitation of individual α subunits, we were able to determine the interaction of each mAchR subtype with the G proteins G_{i1}, G_{i2}, G_{i3}, and $G_{q/11}$.

Experimental Procedures

Materials. Carbachol, poly-D-lysine, and Protein A-Sepharose were from Sigma (Deisenhofen, Germany), [^{35}S]GTPγS (1344 Ci/mmol),

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[2] We refer to the closely related α subunits α_q and α_{11} and to the corresponding G proteins G_q and G_{11} as $\alpha_{q/11}$ and $G_{q/11}$, respectively.

ABBREVIATIONS: mAchR, muscarinic acetylcholine receptor; PTX, pertussis toxin; PLC, phospholipase C; PLD, phospholipase D; HEK, human embryonic kidney; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; QNB, quinuclidinyl benzilate; DMEM, Dulbecco's modified Eagle medium; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

n-[9,10-^{3}H]oleic acid (10 Ci/mmol), *myo*-[2-^{3}H]inositol (24.4 Ci/mmol), [*phenyl*-4-^{3}H]QNB (45.7 Ci/mmol), and [α-^{32}P]GTP (3000 Ci/mmol) were from New England Nuclear (Bad Homburg, Germany), and DMEM/F-12 medium, fetal calf serum, and G418 were from GIBCO BRL (Eggenstein, Germany). PTX was obtained from List (Campbell, CA). Sources of other materials have been described (14).

Cell culture, membrane preparation, and [^{3}H]QNB binding. HEK cells stably expressing human m1, m2, and m3 mAchRs were cultured in DMEM/F-12 growth medium containing 10% (v/v) fetal calf serum, 0.2 unit/ml penicillin, and 0.2 μg/ml streptomycin. The m1, m2, and m3 cell lines express 350,000, 120,000, and 200,000 specific receptors/cell, respectively (5). Stocks of the transfected cell lines were maintained in the presence of the neomycin analogue G418 (0.5 mg/ml). Cells were grown in culture dishes that had been precoated with poly-D-lysine (M_r >300,000, 0.05–0.1 mg/dish) to facilitate attachment of the cells to the plastic surface. For experiments, cells were grown to near-confluence in DMEM/F-12 medium lacking G418. ADP-ribosylation of G_i-type G proteins was performed by adding 100 ng/ml PTX to the medium for the last 16–17 hr of culture. For preparation of membranes, cells were scraped off in phosphate-buffered saline and harvested by centrifugation (10 min at 2000 × *g*). After resuspension in 10 mM triethanolamine-HCl, pH 7.4, 140 mM NaCl, cells were again centrifuged. The final pellet was resuspended in 20 ml of lysis buffer (15), cells were homogenized by nitrogen cavitation, and crude membranes were isolated by differential centrifugation as described before (15). For estimation of mAchR density, binding of 3 nM [^{3}H]QNB to membranes (about 25 μg of protein/tube) was performed in a reaction mixture (500 μl) containing 50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, and 1 mM EDTA. The reaction was started by the addition of membranes to the temperature-equilibrated reaction mixture and was conducted for 60 min at 37°. Separation of membrane-bound and free radioactivity was performed by rapid filtration through glass fiber filters (Whatman GF/C) soaked in washing buffer (50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$) containing 0.33% (v/v) polyethyleneimine. The filters were washed five times with 2 ml of ice-cold washing buffer and were counted in a liquid scintillation counter. Nonspecific binding was defined as the binding not competed for by 1 μM atropine. [^{3}H]QNB binding demonstrated the expression of 8.3, 4.6, and 7.8 pmol/mg of membrane protein for the m1, m2, and m3 mAchRs, respectively.

Phospholipid labeling and measurement of PLC and PLD activity. Cellular phospholipids were labeled by incubating nearly confluent monolayers of cells for 20–24 hr with [^{3}H]oleic acid (2–2.5 μCi/ml) and *myo*-[^{3}H]inositol (2.5 μCi/ml) in growth medium. Incubations were performed as described before (7). The reactions were stopped by addition of 1 ml of ice-cold methanol to the dishes. The cells were scraped off the dishes, and the phospholipids and inositol phosphates were extracted and analyzed as described (7).

GTPγS binding assay. Binding of 0.4–0.7 nM [^{35}S]GTPγS to HEK cell membranes was performed in a reaction mixture (100 μl) containing 50 mM triethanolamine-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, and the indicated additions. The reaction was started by the addition of membranes (about 5 μg of protein/tube) to the temperature-equilibrated reaction mixture and was conducted for 60 min at 30°. Separation of membrane-bound and free radioactivity was performed by rapid filtration through glass fiber filters (Whatman GF/C). The filters were washed five times with 2.5 ml each of an ice-cold buffer (50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$) and counted in a liquid scintillation counter. Nonspecific binding was defined as the binding not competed for by 10 μM unlabeled GTPγS. For comparison of mAchR-stimulated binding of [^{35}S]GTPγS in different cell lines, receptor-stimulated binding is given as the carbachol-induced increase, in femtomoles of [^{35}S]GTPγS bound/femtomoles of mAchR expressed.

Photolabeling of membrane proteins. [α-^{32}P]GTP azidoanilide was synthesized and purified as described (14). Cell membranes (50–200 μg of protein/assay tube) were incubated at 30° in buffer containing 0.1 mM EDTA, 5 mM $MgCl_2$, 30 mM NaCl, 1 mM benzamidine, 5 μM GDP, and 50 mM HEPES-NaOH, pH 7.4. After 3 min of preincubation in the absence or presence of receptor agonists, samples were incubated for another 5 min with 10–20 nM [α-^{32}P]GTP azidoanilide (3.5 μCi/tube). The final assay volume was 60 μl. For photolabeling of $G_{q/11}$ α subunits, GDP was omitted from the incubation buffer. After the reaction was stopped by cooling of the samples on ice, samples were centrifuged at 4° for 5 min at 12,000 × *g*, and pellets were resuspended in 60 μl of the aforementioned buffer supplemented with 2 mM glutathione and devoid of GDP. Suspended membranes were then irradiated for 10 sec at 4° with a 254-nm UV lamp (Vilber Lourmat, Torcy, France).

Immunoprecipitation. For immunoprecipitation, photolabeled membranes were pelleted and solubilized in 40 μl of 2% (w/v) SDS at room temperature. Thereafter, 120 μl of precipitation buffer (1%, w/v, Nonidet P-40, 1%, w/v, desoxycholate, 0.5%, w/v, SDS, 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 mM Tris·HCl, pH 7.4) were added. Solubilized membranes were centrifuged at 4° for 10 min at 12,000 × *g* to remove insoluble material, and 50 μl of 10% (w/v) Protein A-Sepharose beads were added for preclearing of the lysates. After 1 hr, Sepharose beads were removed by centrifugation, and 15 μl of antisera were added to the supernatants. After incubation of samples at 4° for 2 hr, with constant rotation, 60 μl of 10% (w/v) Protein A-Sepharose beads were added, and samples were incubated overnight. Sepharose beads were pelleted (12,000 × *g* for 3 min) and washed twice with 1 ml of washing buffer A (600 mM NaCl, 50 mM Tris·HCl, pH 7.4, 0.5%, w/v, SDS, 1%, w/v, Nonidet P-40) and twice with washing buffer B (300 mM NaCl, 10 mM EDTA, 100 mM Tris·HCl, pH 7.4). Preparation of samples for SDS-PAGE or counting of precipitated radioactivity was performed as described (16).

SDS-PAGE, immunoblotting, and antisera. SDS-PAGE of photolabeled proteins was performed on 8% (w/v) acrylamide gels containing 4.3 M urea, according to the method of Laemmli (17). Photolabeled membrane proteins were visualized by autoradiography of the dried gels with Kodak X-OMAT AR-5 films. Blotting of membrane proteins separated by SDS-PAGE, processing of immunoblots, and detection of immunoreactive proteins by a chemiluminescence procedure (Enhanced Chemluminescence System (ECL), Amersham, Braunschweig, Germany) have been described (18). Antisera against G protein α subunits were obtained after injection of synthetic peptides corresponding to specific regions of α subunits, coupled to keyhole limpet hemocyanin, into rabbits (19). Sequences of peptides against which the antisera specific for α_{i1} (AS 190), α_{i2} (AS 269), α_{i3} (AS 105), α_s (AS 348), and $\alpha_{q/11}$ (AS 368), as well as antisera specific for α_{common} (AS 8) and $\alpha_{i\ common}$ (AS 266), were raised are shown in Table 1. Specificity of antisera was controlled by testing their selectivity for α subunits expressed in *Escherichia coli*. The antiserum raised against the carboxyl

TABLE 1

Peptide antisera used for immunoprecipitation of G protein α subunits

Antiserum	Peptide sequence[a]	G protein α subunit recognized	Amino acids
α_{common} (AS 8)	(C) GAGESGKSTIVKQMK	$\alpha_{i1/2/3}$, $\alpha_{o1/2}$	40–54[b]
$\alpha_{icommon}$ (AS 266)	(C) NLREDGEKAAREV	$\alpha_{i1/2/3}$	22–34[c]
α_{i1} (AS 190)	(C) LDRIAQPNYI	α_{i1}	159–168[c]
α_{i2} (AS 269)	(C) TGANKYDEAAS	α_{i2}	295–305[b]
α_{i3} (AS 105)	(C) LDRISQSNYI	α_{i3}	159–168[b]
$\alpha_{q/11}$ (AS 368)	(C) LQLNLKEYNLV	$\alpha_{q/11}$	349–359[d]
α_s (AS 348)	(C) RMHLRQYELL	α_s	385–394[c]

[a] (C), an amino-terminal cysteine was added to the original peptide sequence to facilitate coupling to keyhole limpet hemocyanin.

[b] Ref. 44.

[c] Ref. 45.

[d] Ref. 46.

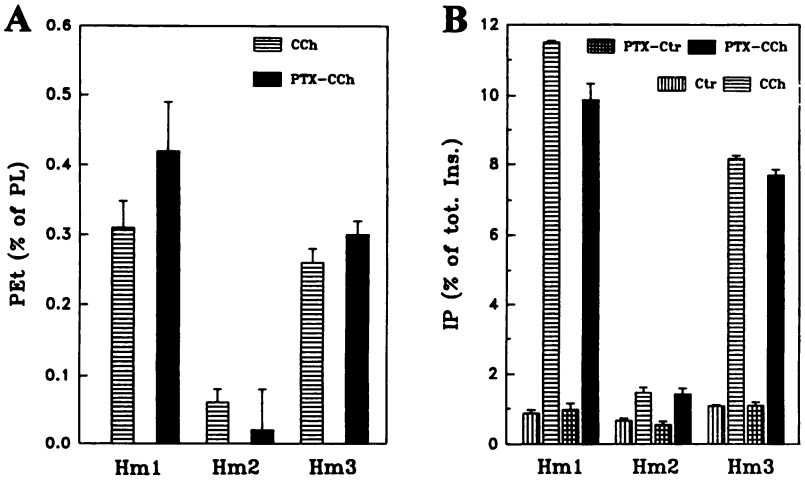

Fig. 1. Carbachol-induced stimulation of PLD and PLC in transfected HEK cells. Untreated and PTX-treated HEK cells expressing human m1, m2, and m3 mAchRs (*Hm1*, *Hm2*, and *Hm3*, respectively) were prelabeled with [^{3}H] oleic acid (A) or [^{3}H]inositol (B) as described in Experimental Procedures. Cells were then incubated for 10 min with 1 mM carbachol (*CCh*). Shown is the formation of [^{3}H] phosphatidylethanol (*PEt*) (A) and [^{3}H]inositol phosphate (*IP*) (B) as a percentage of the total phospholipid (*PL*) and inositol phosphates (*Ins.*), respectively. Shown are mean ± standard deviation values of triplicates. *Ctr*, control.

terminus of $\alpha_{q/11}$ (AS 368) exhibited some cross-reactivity towards recombinantly expressed α_{14}.[3]

Miscellaneous methods and reproducibility. Protein concentration was determined using the bicinchoninc acid protein assay system (Pierce, Rockford, IL). The experiments shown are representative of at least two independently performed experiments, using different membrane preparations.

Results

Incubation of HEK cells expressing m1, m2, and m3 mAchRs and prelabeled with [^{3}H]oleic acid and [^{3}H]inositol in the presence of carbachol resulted in pronounced stimulation of inositol phosphate and phosphatidylethanol formation, as markers for PLC and PLD activity, respectively (Fig. 1). In cells transfected with the m2 receptor, only small increases in PLC and PLD activity could be observed after stimulation with carbachol. This is in agreement with previous results, which in addition showed that PLC stimulation was PTX insensitive (5, 7, 8). Fig. 1A demonstrates that stimulation of PLD also occurred in a PTX-insensitive manner, indicating that stimulation of both enzyme activities via mAchRs involves PTX-insensitive G proteins.

To characterize the G proteins activated by m1, m2 and m3 mAchRs, we first measured the effect of carbachol on binding of [^{35}S]GTPγS to membranes from cells expressing the individual receptor subtypes. In the absence of GDP, carbachol very effectively stimulated [^{35}S]GTPγS binding to membranes from cells transfected with m1 and m3 receptors. In membranes from m2 receptor-transfected cells, the effect of carbachol was small (Fig. 2). In the presence of 1 μM GDP, carbachol was most effective in stimulating binding of [^{35}S]GTPγS via the m2 mAchR, whereas activation of m1 and m3 mAchRs resulted in a significantly smaller increase in [^{35}S]GTPγS binding. These different effects of GDP on stimulation of [^{35}S]GTPγS binding via distinct mAchR subtypes is most likely due to the involvement of different G proteins. As has been shown, effective agonist-dependent binding of hydrolysis-resistant GTP analogues to α subunits of G_i and G_o proteins can more readily be measured in the presence of GDP (20, 21), whereas G proteins of the G_q, G_{12}, and G_s families are effectively activated in the absence of GDP (22, 23). Coupling of activated mAchR subtypes to different G proteins is also indicated by the effect of PTX pretreatment on the ability of carbachol to stimulate [^{35}S]GTPγS binding (Fig. 2). Stimulation of GTPγS binding via m1 and m3 receptors was at least partially insensitive to PTX. In contrast, GTPγS binding stimulated by the activated m2 receptor in the presence of 1 μM GDP was totally PTX sensitive. The small stimulatory effect mediated by the m2 receptor in the absence of GDP appeared to be PTX insensitive.

Fig. 3 shows the effect of carbachol at increasing concentrations on the binding of [^{35}S]GTPγS to membranes from cells transfected with m1, m2, and m3 receptors. In the presence of 1 μM GDP (Fig. 3A), carbachol induced binding of GTPγS via all three receptor subtypes. Carbachol was more effective and appeared to be slightly more potent in stimulating binding via the m2 receptor than via m1 or m3 receptors. At very low GDP concentrations (10 nM) (Fig. 3B), stimulation of GTPγS binding via the m2 receptor was too small for measurement of the concentration dependence of the carbachol effect. However, carbachol acting on m1 and m3 receptors effectively stimulated GTPγS binding, with a potency that appeared to be slightly higher than that in the presence of 1 μM GDP (Fig. 3).

To identify the G proteins activated by agonist-occupied m1, m2, and m3 receptors in transfected HEK cells, we first identified the G protein α subunits expressed in this cell line (Fig. 4). Cell membranes were first photolabeled with [α-^{32}P]GTP azidoanilide in the absence of receptor agonists, separated by SDS-PAGE, and then blotted onto nitrocellulose filters. Filters were exposed to X-ray film and then incubated with different antisera (Table 1), which were visualized by a luminescence procedure (Fig. 4). The anti-α_{common} antiserum recognized several proteins in the range of 40–45 kDa, and an antiserum specific for the α subunits of the G_i-type G proteins, the anti-$\alpha_{i\ common}$ antiserum, detected proteins of 40–41.5 kDa. Accordingly, proteins of 40, 41, and 41.5 kDa were recognized by antisera specific for the α_i subtypes α_{i2}, α_{i3}, and α_{i1}, respectively. Proteins of identical molecular masses were photolabeled with [α-^{32}P]GTP azidoanilide in the absence of receptor agonists and appeared as two bands with the gel system used. The upper band (41/41.5 kDa) comigrated with immunologically identified α_{i3} and α_{i1}, whereas the lower band (40 kDa) comigrated with α_{i2}. In addition, an antiserum raised against a specific sequence common to all α_s subtypes recognized two proteins, of 42.5 and 45 kDa, and an antiserum specific for $\alpha_{q/11}$ reacted with a

[3] K. Spicher, unpublished observations.

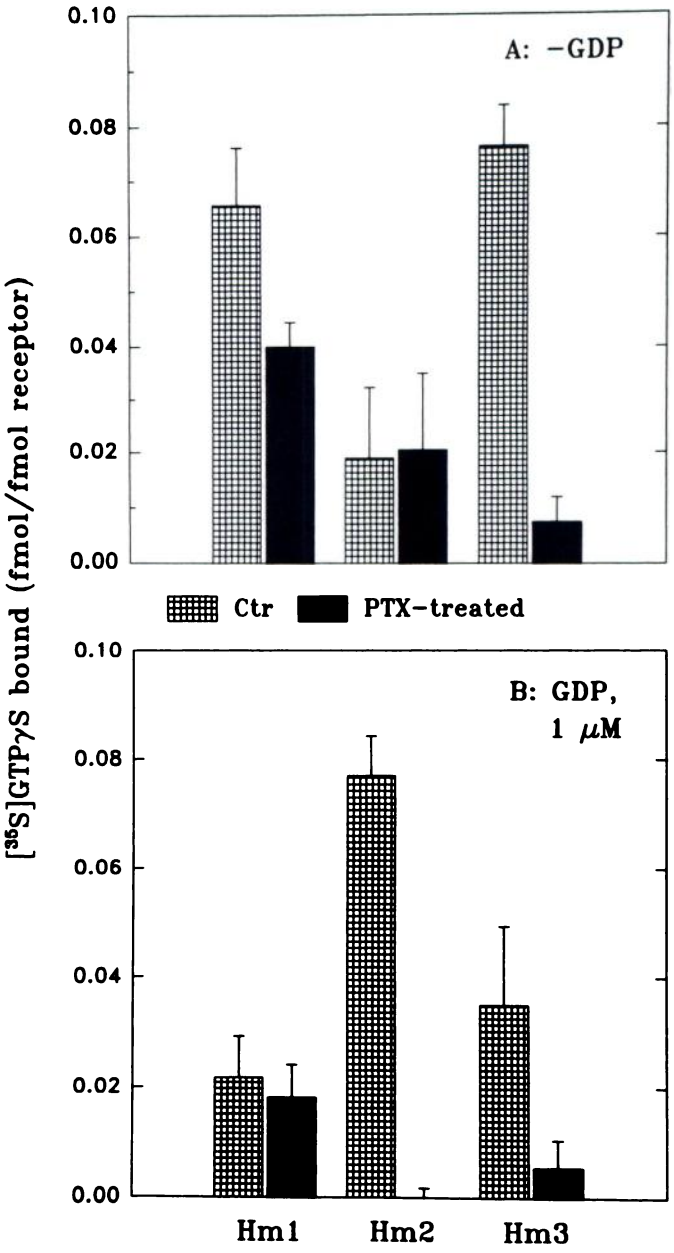

Fig. 2. Stimulation by carbachol of [^{35}S]GTPγS binding in membranes of transfected HEK cells. Membranes from PTX-treated and untreated (*Ctr*) cells were incubated with GTPγS in the absence (A) or presence (B) of 1 μM GDP, without or with 1 mM carbachol, for 60 min. Bound [^{35}S] GTPγS was determined as described in Experimental Procedures. Shown is the carbachol-induced increase (fmol of [^{35}S]GTPγS bound/ fmol of mAchR expressed). Values represent mean values of triplicates. *Hm1*, *Hm2*, and *Hm3*, membranes from HEK cells expressing the human m1, m2, and m3 mAchRs, respectively.

protein of about 42 kDa. We were not able to electrophoretically resolve the α subunits of G_q and G_{11} in membranes of HEK cells. Thus, HEK cells express all three α_i subtypes, $\alpha_{q/11}$, and α_s. Antisera specific for α_{o1} and α_{o2}, as well as for α_{12} and α_{13}, did not recognize corresponding proteins in membranes of HEK cells (data not shown), indicating that these proteins are absent from HEK cells or are expressed at undetectably low levels.

To study the coupling of m1, m2, and m3 mAchRs to individual G proteins, G protein α subunits in membranes of cells expressing each of the receptor subtypes were photolabeled with [α-^{32}P]GTP azidoanilide in the absence or presence of GDP and carbachol, and membrane proteins were subjected to SDS-PAGE. Under these experimental conditions, we found increased photolabeling of proteins comigrating with the G_i-type G proteins in response to receptor activation. Receptor-dependent incorporation of [α-^{32}P]GTP azidoanilide into proteins comigrating with $G_{q/11}$ α subunits was, however, very difficult to determine, because the total amount of photolabel incorporated into these proteins was very small, compared with that found in G_i-type G proteins (data not shown). Therefore, membranes were solubilized after photolabeling, α subunits were immunoprecipitated with selective antisera, and the immunoprecipitates were subjected to SDS-PAGE. Immunoprecipitation with the anti-$\alpha_{i\ common}$ antiserum recognizing all three α_i subtypes revealed an increased incorporation of [α-^{32}P]GTP azidoanilide into proteins of 41/41.5 kDa after activation of m1, m2, and m3 mAchRs by carbachol (Fig. 5). Activation of the m2 subtype additionally led to the increased photolabeling of a 40-kDa protein. In membranes from untransfected HEK cells or from PTX-pretreated transfected HEK cells, no stimulation of photolabeling of α_i proteins was observed (data not shown). Proteins with molecular masses identical to those of proteins immunoprecipitated by the anti-$\alpha_{i\ common}$ antiserum from membranes photolabeled in the presence of carbachol were also recognized by the anti-$\alpha_{i\ common}$ antiserum used in immunoblot experiments and represent the α subunits of G_{i3}/ G_{i1} (41/41.5 kDa) and G_{i2} (40 kDa) (Fig. 4). Thus, it can be assumed that all three receptor subtypes couple to G_{i1} and/or G_{i3} upon activation, whereas the activated m2 mAchR couples to G_{i2} in addition. To test this, we used α_i subtype-specific antisera. Whereas photolabeling of 40-kDa G_{i2} α subunits was effectively stimulated by carbachol acting on m2 receptors, activation of m1 and m3 receptor subtypes was without effect on photolabeling of α_{i2} (Fig. 5). In contrast, carbachol stimulated the incorporation of [α-^{32}P]GTP azidoanilide into 41.5-kDa G_{i1} α subunits, as well as into 41-kDa G_{i3} α subunits, via all three mAchR subtypes (Fig. 6).

Because stimulation of PLC and PLD via m1 and m3 receptors is largely unaffected by PTX (8) (Fig. 1), we tested the effect of activated m1, m2, and m3 receptors on photolabeling of the PTX-insensitive α subunits of $G_{q/11}$, which have been shown to mediate PTX-insensitive stimulation of PLC (24). As shown in Fig. 7, both activated m1 and m3 receptors led to increased photolabeling of 42-kDa $G_{q/11}$ α subunits, whereas carbachol acting on m2 receptors was without effect. As would be expected, very similar results were observed using membranes from PTX-pretreated cells (Fig. 7). No increased photolabeling of G_s α subunits could be observed after immunoprecipitation from membranes photolabeled with the α_s antiserum in the presence of carbachol (data not shown).

Taken together, these data show the selective activation of G_{i1}, G_{i3}, and $G_{q/11}$ via m1 and m3 receptors, whereas activated m2 receptors couple to G_{i1}, G_{i2}, and G_{i3}. To confirm these results and to determine the potency of carbachol to induce G protein activation through m1, m2, and m3 mAchRs, we performed photolabeling experiments in the presence of increasing concentrations of carbachol (Figs. 8 and 9). In membranes of m1 and m3 receptor-expressing cells, carbachol concentration-dependently stimulated photolabeling of the comigrating 41- and 41.5-kDa α subunits of G_{i1} and G_{i3}, which were immunoprecipitated by the anti-$\alpha_{i\ common}$ antiserum, as well as of the 42-kDa protein immunoprecipitated by the anti-$\alpha_{q/11}$ antiserum

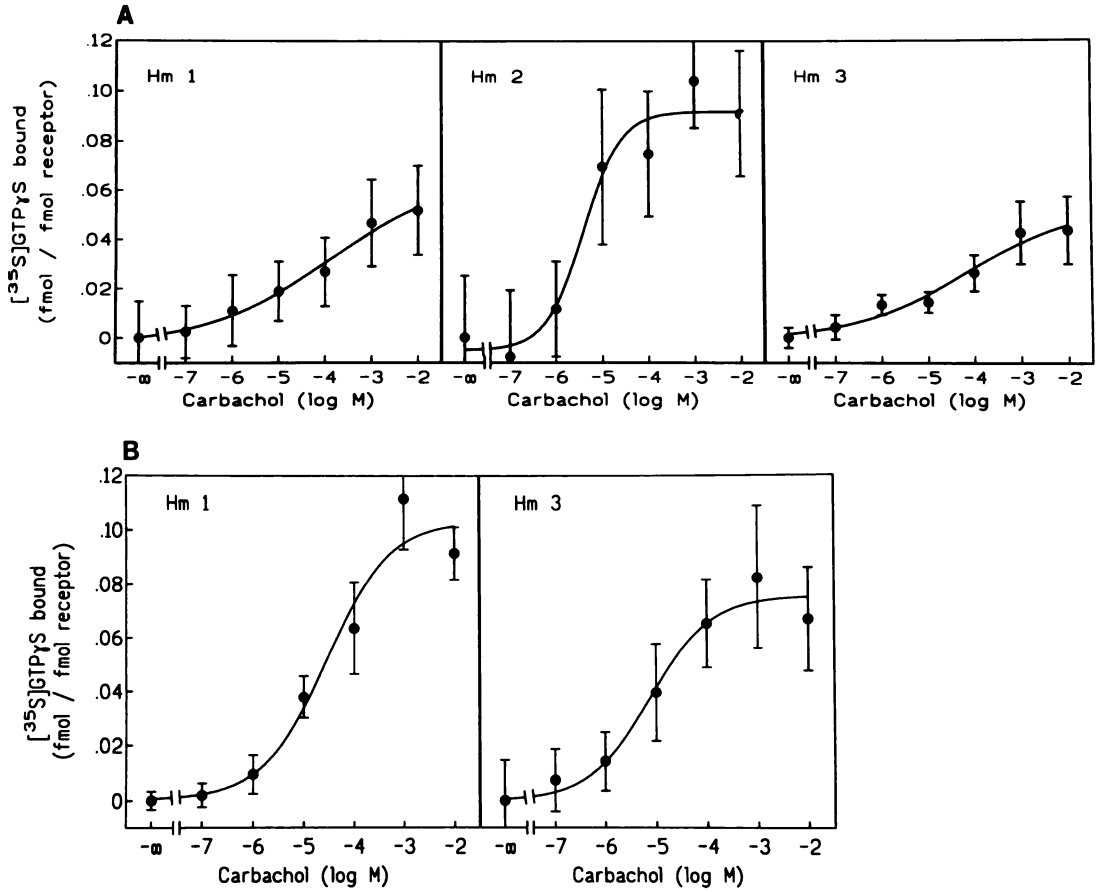

Fig. 3. Concentration dependence of carbachol-induced [^{35}S]GTPγS binding to membranes of transfected HEK cells. Binding of GTPγS was determined in membranes of m1, m2, or m3 receptor-expressing HEK cells (*Hm1*, *Hm2*, and *Hm3*, respectively) that had been incubated for 60 min with increasing concentrations of carbachol in the presence of 1 μM (A) or 10 nM (B) GDP. Bound [^{35}S]GTPγS was determined as described in Experimental Procedures. Shown is the carbachol-induced increase (fmol of [^{35}S]GTPγS bound/fmol of mAchR expressed). Values represent mean values of triplicates.

(Fig. 8, A and B). Incorporation into the 40-kDa band immunoprecipitated by the anti-$\alpha_{i\ common}$ antiserum and representing α_{i2} (Figs. 4 and 5) remained unaffected. Carbachol-induced activation of G_{i1}/G_{i3} via m1 and m3 receptors could be observed at 1 μM carbachol and did not reach a maximum below carbachol concentrations of 1 mM, the highest concentration used. Carbachol appeared to be slightly more potent with regard to stimulation of [α-^{32}P]GTP azidoanilide binding to $\alpha_{q/11}$, which was maximal at agonist concentrations of 10–100 μM. Similar maximally effective concentrations were found for the carbachol-induced photolabeling of α subunits precipitated by the anti-$\alpha_{i\ common}$ antiserum from membranes of m2 receptor-expressing cells (Fig. 8C). Activation of G_i-type G proteins by carbachol acting on m2 receptors could be observed at 10–100 nM carbachol, and there were no obvious differences in the potency of carbachol to stimulate photolabeling of 41/41.5-kDa G_{i3}/G_{i1} α subunits and of 40-kDa G_{i2} α subunits via m2 receptors. For better quantification of the concentration dependence of carbachol-induced m1 and m3 receptor-mediated activation of $G_{q/11}$, autoradiograms showing concentration-dependently photolabeled $\alpha_{q/11}$ were densitometrically evaluated (Fig. 8D). Stimulation of photolabeling of $\alpha_{q/11}$ by carbachol acting on both receptors occurred with half-maximal and maximal concentrations of about 1 μM and 10–100 μM, respectively.

Because the amount of radioactivity incorporated into the α_i subunits immunoprecipitated by the anti-$\alpha_{i\ common}$ antiserum was several times greater than that found in immunoprecipitated $\alpha_{q/11}$ (due to different expression levels of α subunits and different effectiveness of the antisera used), incorporation of [α-^{32}P]GTP azidoanilide into α_i subunits could be quantitated by counting the immunoprecipitated radioactivity (Fig. 9). The maximal concentration of carbachol (1 mM) used produced stimulation of basal binding of [α-^{32}P]GTP azidoanilide to α_i subunits by 145%, 263%, and 169% when acting on m1, m2, and m3 receptors, respectively. Measuring the effect of increasing carbachol concentrations confirmed different potencies for carbachol-induced G_i activation. Whereas carbachol stimulated photolabeling of α_i subunits via m2 receptors with an EC_{50} of about 1 μM, being maximally effective at 100 μM, stimulation via m1 and m3 receptors occurred with EC_{50} values of >10 μM and did not reach a clear saturation at the carbachol concentrations used (up to 1 mM). Thus, on the basis of selective activation of G protein subtypes by different mAchRs, carbachol with comparably high potency led to the activation of all G_i-type G proteins via m2 receptors but was less potent in activating G_{i1}/G_{i3} via m1 and m3 receptors.

Discussion

In the present paper, we used HEK cells expressing m1, m2, and m3 human mAchRs to identify the G proteins coupled to

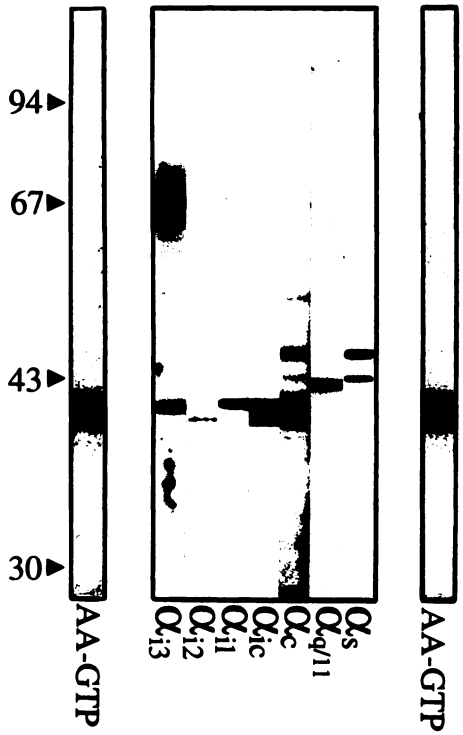

Fig. 4. G protein α subunits in membranes of HEK cells. Membrane proteins were photolabeled with [α-^{32}P]GTP azidoanilide in the absence of GDP as described in Experimental Procedures, separated by SDS-PAGE, and blotted onto nitrocellulose filters. The filters were subjected to autoradiography to detect [α-^{32}P]GTP azidoanilide (*AA-GTP*) and were cut into strips, which were incubated with different antibodies [α_c, anti-α_{common} antiserum (AS 8); α_{ic}, anti-$\alpha_{i\,common}$ antiserum (AS 266); α_{i1}, anti-α_{i1} antiserum (AS 190); α_{i2}, anti-α_{i2} antiserum (AS 269); α_{i3}, anti-α_{i3} antiserum (AS 105); $\alpha_{q/11}$, anti-$\alpha_{q/11}$ antiserum (AS 368); α_s, anti-α_s antiserum (AS 348)]. Bound antibodies were visualized by a chemiluminescence procedure, as described. Shown are autoluminograms of nitrocellulose strips. For better comparison, strips were exposed to X-ray films for different time periods. *Numbers on the left*, molecular masses of marker proteins.

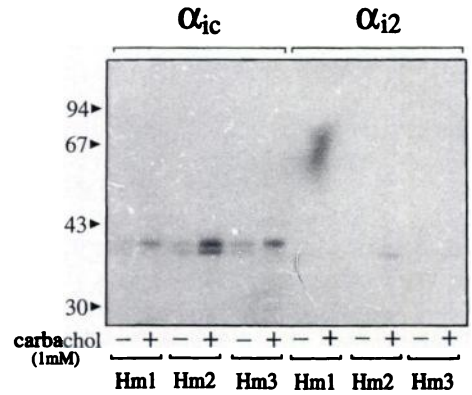

Fig. 5. Carbachol-stimulated photolabeling of G_i α subunits in membranes from transfected HEK cells. Membranes (100 μg of protein/tube) were photolabeled in the absence (*carbachol* −) or presence (*carbachol* +) of 1 mM carbachol, with [α-^{32}P]GTP azidoanilide. Membranes were solubilized and incubated with the indicated antisera [α_{ic}, anti-$\alpha_{i\,common}$ antiserum (AS 266); α_{i2}, anti-α_{i2} antiserum (AS 269)]. Immunoprecipitation was performed as described in Experimental Procedures. Precipitated proteins were subjected to SDS-PAGE. Shown is an autoradiogram of an SDS gel, with the molecular masses of marker proteins on the *left*. *Hm1*, *Hm2*, and *Hm3*, membranes from HEK cells expressing the human m1, m2, and m3 mAchRs, respectively.

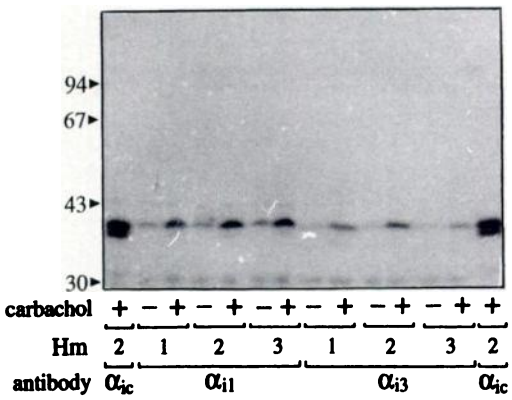

Fig. 6. Carbachol-stimulated photolabeling of G_{i1} and G_{i3} α subunits in membranes from transfected HEK cells. Membranes (100 μg of protein/tube) were photolabeled in the absence (*carbachol* −) or presence (*carbachol* +) of 1 mM carbachol, with [α-^{32}P]GTP azidoanilide. Membranes were solubilized and incubated with the indicated antisera [α_{i1}, anti-α_{i1} antiserum (AS 190); α_{i3}, anti-α_{i3} antiserum (AS 105); α_{ic}, anti-$\alpha_{i\,common}$ antiserum (AS 266)]. Immunoprecipitation was performed as described in Experimental Procedures. Precipitated proteins were subjected to SDS-PAGE. Shown is an autoradiogram of an SDS gel, with the molecular masses of marker proteins on the *left*. *Hm1*, *Hm2*, and *Hm3*, membranes from HEK cells transfected with the human m1, m2, and m3 mAchRs, respectively.

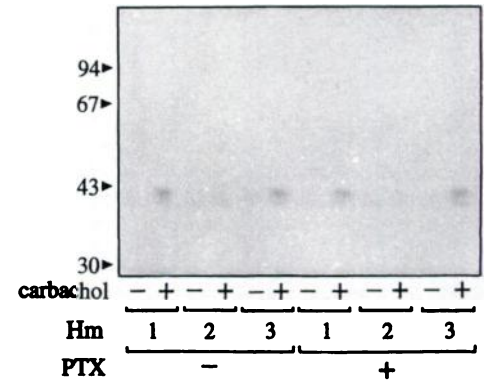

Fig. 7. Carbachol-stimulated photolabeling of $G_{q/11}$ α subunits in membranes from transfected HEK cells. Membranes (200 μg of protein/tube) from untreated (*PTX* −) or PTX-treated (*PTX* +) cells were photolabeled in the absence (*carbachol* −) or presence (*carbachol* +) of 1 mM carbachol, with [α-^{32}P]GTP azidoanilide. Membranes were solubilized and incubated with the anti-$\alpha_{q/11}$ antiserum (AS 368). Immunoprecipitation was performed as described. Precipitated proteins were subjected to SDS-PAGE. Shown is an autoradiogram of an SDS gel, with the molecular masses of marker proteins on the *left*. *Hm1*, *Hm2*, and *Hm3*, membranes from HEK cells expressing the human m1, m2, and m3 mAchRs, respectively.

each of these receptor subtypes. HEK cells have been successfully used to determine the effector pathways regulated by the cloned human mAchRs (5, 7) and therefore may also represent a suitable system for a comparative study of the interaction of particular mAchRs and G protein subtypes within the same cell type. In the latter case, differences in receptor-G protein coupling can be attributed only to the receptor subtype and are not influenced by cell type-specific variations.

Probing of membrane proteins from HEK cells with specific antisera identified the three known subtypes of G_i α subunits (α_{i1}, α_{i2}, and α_{i3}), $\alpha_{q/11}$, and at least two forms of α_s (Fig. 4). To characterize the coupling of molecularly defined mAchRs and distinct G proteins in plasma membranes, we photolabeled with [α-^{32}P]GTP azidoanilide the α subunits of the G proteins

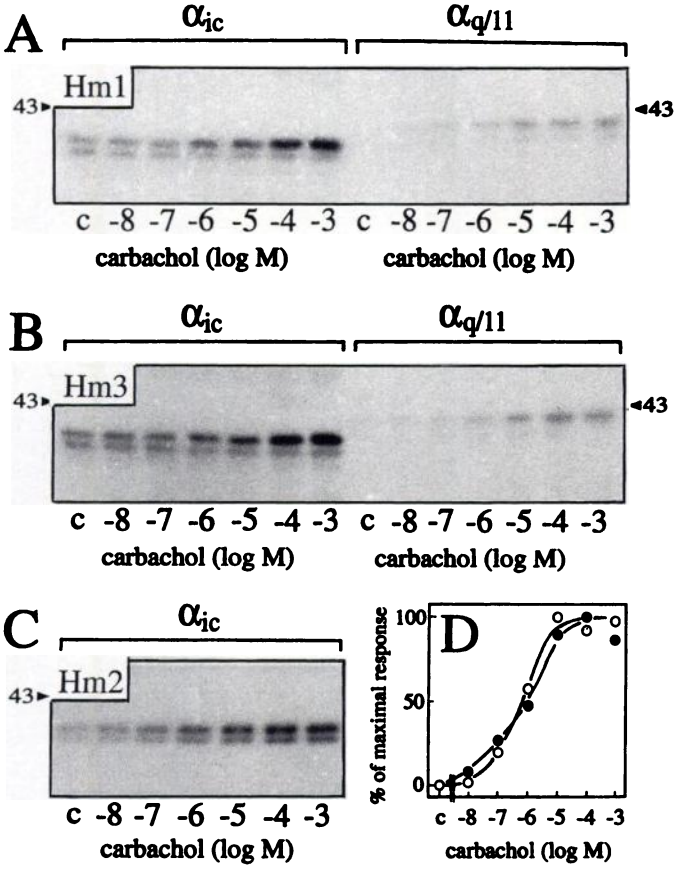

Fig. 8. Concentration dependence of carbachol-induced photolabeling of G protein α subunits in transfected HEK cells. Membranes were incubated with carbachol at increasing concentrations as indicated and were photolabeled with [α-^{32}P]GTP azidoanilide. Solubilized membranes were immunoprecipitated with the indicated antisera [α_{ic}, anti-$\alpha_{i\ common}$ antiserum (AS 266); $\alpha_{q/11}$, anti-$\alpha_{q/11}$ antiserum (AS 368)] as described, and precipitated proteins were subjected to SDS-PAGE. Shown are the 35–46-kDa regions from autoradiograms of SDS gels. For precipitation with the anti-$\alpha_{i\ common}$ and anti-$\alpha_{q/11}$ antisera, 100 and 200 μg of membranes were used, respectively. *Hm1* (A), *Hm3* (B), and *Hm2* (C), membranes from HEK cells transfected with the human m1, m3, and m2 mAchRs, respectively. *Arrowheads on the left and right*, position of the 43-kDa marker protein. D, Membranes from m1 (○) and m3 (●) receptor-expressing HEK cells were photolabeled in the presence of increasing carbachol concentrations (*abscissa*) and immunoprecipitated with the anti-$\alpha_{q/11}$ antiserum (AS 368). Shown is the densitrometric evaluation of the 41–43-kDa region of resulting autoradiograms, as a percentage of the maximal stimulation. *c*, control (without carbachol).

activated via individual receptor subtypes and then isolated the α subunits by immunoprecipitation with antisera directed against specific regions of the G protein α subunits. This approach has been used to determine the interaction of different receptors with PTX-insensitive and -sensitive G proteins within their natural membrane environment (16, 22).

We could show that m1, m2, and m3 mAchRs coupled selectively with distinct G proteins when expressed in HEK cells (Figs. 5–7). Whereas carbachol selectively activated the PTX-insensitive G proteins $G_{q/11}$ when acting through m1 and m3 receptors, G_{i2} was selectively activated via m2 receptors and all three mAchR subtypes studied mediated activation of G_{i1}/G_{i3}. However, activation of G_{i1}/G_{i3} by carbachol-activated m1 and m3 receptors occurred at higher agonist concentrations (EC_{50} of about 10–20 μM) than did m2 receptor-mediated G_{i1}/G_{i3} activation (EC_{50} of about 1 μM) (Figs. 8 and 9). This is unlikely

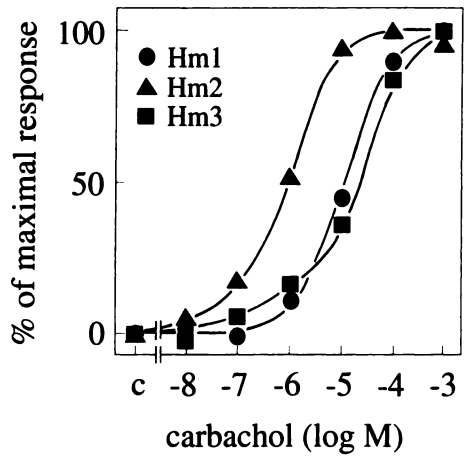

Fig. 9. Effect of carbachol at increasing concentrations on incorporation of [α-^{32}P]GTP azidoanilide into G_i α subunits in membranes from transfected HEK cells. Membranes (200 μg of protein/tube) from cells expressing the human m1 (●), m2 (▲), and m3 (■) mAchRs were incubated with increasing concentrations of carbachol and photolabeled with [α-^{32}P]GTP azidoanilide as described. Membranes were solubilized and immunoprecipitated with the anti-$\alpha_{i\ common}$ antiserum (AS 266). The immunoprecipitates were counted for radioactivity, and radioactivity precipitated by preimmune serum was subtracted from the radioactivity precipitated by the anti-$\alpha_{i\ common}$ antiserum. Shown is the stimulation of photolabeling, as a percentage of the maximal stimulation. Values are mean values of triplicates.

to reflect the slightly lower affinity of carbachol for m1 and m3 receptors, compared with its affinity for m2 receptors (2), and is also unlikely to be due to a failure of the transfected receptors to couple to G proteins, because both m1 and m3 receptors, in contrast to the m2 receptor, effectively mediated the activation of $G_{q/11}$ by carbachol (Figs. 7 and 8).

It is, however, not clear whether G_{i1}/G_{i3} activation via m1 and m3 receptors represents a physiological phenomenon. Taking into account the fact that transfected cells express relatively high levels of receptors, the high receptor occupancy at high agonist concentrations may lead to increased promiscuity of the receptor-G protein interaction, resulting in the activation of G proteins that are normally not activated by this receptor. If this is the case, then it remains remarkable that even at high agonist concentrations no coupling of m1 and m3 receptors to G_{i2} and of m2 receptors to $G_{q/11}$ was found. In any case, our data demonstrate that m1 and m3 receptors have the potential to activate G_{i1} and G_{i3}. This explains several findings showing PTX-sensitive effects in response to m1 or m3 receptor activation (25–28), including the PTX-sensitive portion of PLC activation induced by 1 mM carbachol in membranes from CHO cells expressing these receptor subtypes (8). The PTX-insensitive PLC stimulation via m1 and m3 receptors is most likely mediated by $G_{q/11}$ (24). Consistent with this, activation of $G_{q/11}$ by carbachol was found only in membranes from m1 and m3 receptor-expressing cells and occurred with EC_{50} values (1 μM) (Fig. 8) very similar to those reported for m1 and m3 receptor-mediated activation of PLC stimulation in HEK cells (1 and 1.6 μM, respectively) (5). Activated m2 receptors also lead to increased PLC activity. This effect, however, is very small and displays a severalfold higher EC_{50} than that induced by m1 and m3 receptors (5). Fig. 1 shows that this effect occurred in a PTX-insensitive manner, indicating the involvement of PTX-insensitive G proteins. As mentioned above, no activation of $G_{q/11}$, the main PTX-insensitive G protein found in HEK cell

membranes, could be observed (Fig. 7). Thus, either another PTX-insensitive G protein mediates this effect or, as appears more likely, the small amount of activated $G_{q/11}$ underlying the small m2 receptor-induced PLC activation escapes experimental detection. Interestingly, the m2 receptor expressed in CHO cells leads to an effective PTX-sensitive stimulation of PLC. This PTX-sensitive portion of PLC activation may result from $\beta\gamma$ subunits released from activated G_i-type G proteins, which have been shown to stimulate β isoforms of PLC (29, 30). The lack of PTX-sensitive activation of PLC in HEK cells via the m2 receptor points to cell type-specific characteristics that result in different mechanisms of receptor-effector coupling, as shown for dopamine D_2 receptors and α_2-adrenoceptors (31, 32). The mechanism of PLD regulation by G protein-coupled receptors is still a matter of debate. Stimulation of PLD via m1, m2, and m3 receptors in HEK cells resembled stimulation of PLC by each of the activated receptors, with regard to its efficiency as well as its sensitivity to PTX (Fig. 1). This is in line with the concept that the mechanisms of activation of the two enzymes are closely related.

Among the studied mAchRs, only the m2 receptor subtype mediated activation of G_{i2} (Fig. 5). Even at high levels of receptor occupancy m1 and m3 receptors did not couple to G_{i2}, whereas they effectively activated G_{i1} and G_{i3} under these conditions (Figs. 6 and 8). This finding raises the question of whether G_{i2} is involved in the PTX-sensitive regulation of an effector that occurs exclusively in response to m2 receptor activation. The m2 receptor has been shown to lead to PTX-sensitive inhibition of adenylyl cyclase in different transfected cell types (5, 10). In contrast, both the m1 and m3 receptors concentration-dependently increased the adenylyl cyclase activity in HEK cells (5), an effect thought to be secondary to PLC activation by m1 and m3 receptors (33). This stimulatory effect on cAMP formation remains unchanged even at high agonist concentrations (5), which lead to G_{i1}/G_{i3} activation (Figs. 6 and 8), suggesting that G_{i1} and G_{i3}, in contrast to G_{i2}, do not mediate receptor-dependent inhibition of adenylyl cyclase in HEK cells. This is in contradiction to data based on selective carboxyl-terminal anti-α_i subtype antibodies (34, 35), as well as data obtained by expression of mutationally activated α_i subunits and reconstitution studies with recombinant proteins, which show that all three G_i subtypes are capable of adenylyl cyclase inhibition (36, 37). However, there are also several reports demonstrating that inhibition of adenylyl cyclase by different receptors under *in situ* conditions involves G_{i2}, but not G_{i1}/G_{i3} (38–41). The inhibitory regulation of adenylyl cyclase appears to be much more complicated, because $\beta\gamma$ complexes of G proteins, which in different combinations may associate with α subunits and determine coupling of the G protein heterotrimer with receptors, can also induce the inhibition of certain isoforms of adenylyl cyclase (42, 43). Nevertheless, our data would be in agreement with a primary role of G_{i2} in receptor-dependent adenylyl cyclase inhibition, whereas the functional role of m1 and m3 receptor-mediated activation of G_{i1} and G_{i3} needs further clarification.

Taken together, our data demonstrate the selective coupling of mAchR subtypes to certain G proteins and principally confirm the concept that activated m2 receptors efficaciously couple to PTX-sensitive G proteins, whereas activated m1 and m3 receptors efficaciously couple to PTX-insensitive G proteins like $G_{q/11}$ in intact plasma membranes. In addition, m1 and m3 receptors possess the potential to couple to the G_{i1} and G_{i3} subtypes of the G_i family.

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