0026-895X/91/060908-07$03.00/0

MOLECULAR PHARMACOLOGY, 40:908-914

Subtype-Specific Antibodies for Muscarinic Cholinergic Receptors. II. Studies with Reconstituted Chick Heart Receptors and the GTP-Binding Protein G_o

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Received January 14, 1991; Accepted August 12, 1991

SUMMARY

The antibodies described in the accompanying paper were used to probe the interactions of the chick heart muscarinic acetylcholine receptors (mAChRs) with the GTP-binding protein G_o. The anti-m4b antibodies, which were made against a peptide from the amino-terminal portion of the third cytoplasmic loop of the m4 mAChR subtype, were tested for their abilities to affect the coupling of the chick heart mAChR to the GTP-binding protein G_o. The purified chick heart mAChRs were reconstituted with purified G_o in phospholipid vesicles, and their interactions were monitored in the presence or absence of the antibodies. The anti-m4b antibodies completely inhibited the ability of G_o to promote high affinity agonist binding to the purified receptors. The anti-m4b antibodies also completely inhibited the agonist-stimulated binding of guanosine-5′-*O*-(3-thio)triphosphate (GTPγS) to G_o and the receptor-stimulated GTPase activity of G_o. These findings indicate that the amino-terminal portion of the third cytoplasmic loop is an important determinant for G_o to promote high affinity agonist binding to the chick heart mAChR and also for the agonist-stimulated GTPγS binding and GTPase activity. The anti-m4a, anti-m2, and anti-m1a antibodies, which were made against centrally located peptides of the third cytoplasmic loop of the m4, m2, and m1 mAChR subtypes, respectively, were also tested for their effects in the reconstituted receptor/G_o system. The anti-m2 and anti-m4a antibodies also significantly reduced agonist-stimulated GTPγS binding, as well as GTPase activity, but did not completely abolish these functions, as was the case with anti-m4b antibodies. However, the anti-m4a and anti-m2 antibodies shared with anti-m4b antibodies the ability to markedly inhibit the ability of G_o to promote high affinity agonist binding to the purified and reconstituted receptors. In contrast to the results obtained with the anti-m2 and anti-m4 antibodies, the anti-m1a antibodies had smaller effects on the receptor/G_o interactions. These results suggested that central portions of the loop can also influence mAChR/G_o interactions. Studies were also performed to test the effects of the peptides that were used as antigens on receptor-mediated GTPγS binding to G_o. Each of the peptides caused significant inhibition of this function, but the greatest inhibition was observed with the m4b peptide. In sum, the results suggest that multiple domains in the third cytoplasmic loop of chick heart mAChR can modulate interactions with G_o.

mAChRs produce their effects by interacting with G proteins that couple the receptors to effectors. Studies of signal transduction mediated by mAChRs have revealed that these receptors are capable of interacting with a number of different G proteins, including (i) G_i, which attenuates adenylyl cyclase and in some cells can stimulate PI hydrolysis (1, 2), (ii) G_o, which also can stimulate PI hydrolysis in some cells and may have other functions, and (iii) G_p, a pertussis toxin-insensitive G protein that couples the receptors to stimulation of PI hydrolysis in many cells (3–5). The factors determining the specificity and efficiency of mAChR/G protein interaction have begun to be defined but remain largely unknown. Recent studies using deletion mutants of the mAChR and chimeric mAChRs have indicated that the third cytoplasmic domain of these receptors plays a central role in mAChR/G protein interactions (6–9).

We have used antibodies (described in the accompanying paper) prepared against different regions of the third intracellular loops of the mAChR to study the interactions of the chick heart mAChR with G_o. The results in the accompanying paper demonstrated that both the anti-m4 and anti-m2 antibodies recognized the predominant mAChR in chick heart. In view of these results, we reasoned that these antibodies could be useful in the study of chick heart mAChR/G protein interaction. In the studies reported here, three antibodies against different portions of the third cytoplasmic loop of m2 and m4 mAChR were used, i.e., the anti-m4b antibody, which was made against

This work was supported by National Institutes of Health Grant HL 31601. R.M.R. is the recipient of a fellowship from the American Heart Association of Metropolitan Chicago.

ABBREVIATIONS: mAChR, muscarinic acetylcholine receptor; G protein, GTP-binding protein; QNB, quinuclidinyl benzilate; PI, phosphoinositide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

peptide from the amino-terminal region of the third loop of the m4 mAChR, and anti-m4a and anti-m2 antibodies, which were made against peptides from the centrally located domains of the third loop of m4 and m2 mAChRs. These antibodies were tested for effects on the ability of G_o to restore high affinity agonist binding to the purified mAChR, to modify the agonist-stimulated binding of GTPγS to G_o, and to modulate receptor-stimulated GTPase activity of G_o.

Materials and Methods

Purification of mAChRs and G proteins. mAChRs were purified from chick heart ventricles, to a specific activity of ~1 nmol of [^{3}H]QNB bound/mg of protein, using the procedure of Haga and Haga (10) as described by Kwatra and Hosey (11). Although the receptors appeared to be >90% pure by silver staining, as noted previously by ourselves and others (10–12), the specific activity of the purified receptors was lower than the predicted theoretical specific activity. The reasons for this are unclear (see Ref. 12 for discussion). G proteins were purified from calf brain according to the procedure described by Sternweis and Robishaw (13), which resulted in the purification of a mixture of heterotrimeric G_o and G_i proteins, in the ratio of 80:20. G_o was further purified from the G_o/G_i mixture by the use of Mono-Q column chromatography, as described by Katada *et al.* (14). The specific activity of the purified G_o was 14 nmol of GTPγS bound/mg of protein.

Ligand binding assays with reconstituted mAChR and G_o. Reconstitution of mAChR with G_o was performed as described by Haga *et al.* (15), using a ratio of G_o to receptor of 250:1. Before reconstitution with G_o, some preparations of receptors were preincubated for 2–3 hr, on ice, either with 0.3 μg of antibody/0.16–0.20 pmol of purified receptor or with nonimmune serum or antibody preadsorbed with 50 μg/ml m4b peptide. Before use, the antibody or the peptide-preadsorbed antibody was dialyzed extensively against buffer containing 20 mM HEPES, pH 8.0, 0.1 mM EDTA, 0.1 M NaCl, and 5 mM dithiothreitol. Ligand binding to the reconstituted mAChR was carried out with the antagonist [^{3}H]QNB and varying concentrations of the agonist carbachol, as previously described (16, 17). Briefly, the assay (1 ml) contained 40–50 fmol of reconstituted receptors with or without G_o, 25 mM KPO_4, pH 7.0, 0.8 mM EDTA, 3 mM $MgCl_2$, 230 mM NaCl, 0.06% bovine serum albumin, 4 mM HEPES, and 0.6–0.7 nM [^{3}H]QNB. Reactions were carried out for 75 min at 30° and terminated by filtration over GF/F filters.

[^{35}S]GTPγS binding assays. Vesicles containing purified mAChR were preincubated in the presence or absence of antibody, as described above. The receptors were then reconstituted with G_o, using 5 pmol of G_o/pmol of purified receptor, for 1 hr on ice. Before the [^{35}S]GTPγS binding assays were performed, the aforementioned reconstitution mixtures were first incubated for 5 min on ice with unlabeled GDP (5 μM final concentration), because GDP is known to suppress basal binding of GTPγS but allows stimulation by agonist (18). Reactions were carried out in a final volume of 40 μl of a solution containing 25 mM sodium HEPES, pH 8.0, 1 mM EDTA, 0.5 mM dithiothreitol, 3 mM $MgCl_2$, 100 mM NaCl, 0.1% Lubrol, and [^{35}S]GTPγS (200,000 cpm/assay tube), with carbachol (0.1 mM), with or without atropine (0.2 mM), for 0–30 min at 30°. The reactions were terminated by addition of 2 ml of cold washing buffer containing 20 mM Tris·HCl, pH 8.0, 100 mM NaCl, and 25 mM $MgCl_2$. The bound [^{35}S]GTPγS was trapped on nitrocellulose filters (type HA, 0.45 μm; Millipore Corporation, Bedford, MA) and counted in a liquid scintillation counter.

GTPase assays. Incubation of the reconstituted receptor with antibody or nonimmune sera and the reconstitution of the receptor with G_o were performed as described above for the GTPγS binding assays. GTPase activity was assayed essentially according to the procedure described by Cerione *et al.* (19). Briefly, 100 μl of assay volume contained 40 μl of receptor/G_o-containing vesicles and 10 mM Tris·HCl, pH 7.8, 3 mM $MgCl_2$, 1 mM EDTA, 2 mg/ml bovine serum albumin, 0.5 mM ascorbic acid, 100–200 nM [γ-^{32}P]GTP (10,000–40,000 cpm/pmol; ICN), 0.1 mM carbachol, with or without 0.2 mM atropine or 1 mM unlabeled GTP. The reaction mixtures were incubated at 30° for 30–60 min. The reactions were stopped by addition of 10 μl of cold 50% trichloroacetic acid, with immediate chilling on ice. The mixtures were centrifuged for 10 min at 2500 rpm, and then 90 μl of the supernatant were removed and assayed for inorganic [^{32}P]phosphate, as previously described (19).

Results

Effect of antibodies to modulate the ability of G_o to induce high affinity agonist binding to purified chick heart mAChRs. Recent studies have provided compelling evidence that the amino-terminal region of the third cytoplasmic domain of the mAChR is important in the coupling of mAChR to G proteins (7–9). Therefore, it might be expected that the anti-m4b antibodies (described in the accompanying paper), which were made against the amino-terminal region of the third cytoplasmic loop of the m4 mAChR, might have functional effects on receptor/G protein coupling. To test this concept, we assessed the effects of the anti-m4b antibodies on the interactions of the purified chick heart mAChR with purified G_o in a reconstituted system. Agonist competition studies were performed to evaluate the effects of the anti-m4b antibodies on the ability of G_o to restore high affinity agonist binding to the purified mAChR in reconstituted phospholipid vesicles. In the absence of G_o, receptors exhibited a single low affinity state for carbachol, with a dissociation constant of ~100 μM (Fig. 1; Table 1). The dose-response relationships were best fit to a one-state model (Fig. 1A; Table 1). The addition of G_o to vesicles containing the purified receptors restored high affinity agonist binding to the control receptors (Fig. 1A), and the data were best fit to a two-state model. Guanine nucleotides reversed the ability of G_o to induce high affinity binding (data not shown, but see Ref. 17). The preincubation of the receptors with the anti-m4b antibodies in the absence of G_o did not perturb receptor properties; however, these antibodies totally prevented the G_o-induced formation of the high affinity state of the receptor (Fig. 1B), and only the low affinity state was observed (Table 1). Nonimmune serum or antibodies preadsorbed with peptide did not prevent the receptor/G protein coupling (Fig. 1F); the results obtained under these conditions were similar to those of the control receptors in the presence of G_o (Table 1). These results demonstrated that the antibodies generated against the amino-terminal portion of the third cytoplasmic loop of the m4 mAChR were able to prevent the interaction of G_o with the chick heart mAChR that results in the restoration of high affinity agonist binding.

If additional domains within the third cytoplasmic loop are involved in defining the efficiency of the receptor/G protein coupling, then antibodies against other stretches of this loop might also affect the ability of G_o to promote high affinity binding to the purified chick heart mAChR. Therefore, effects of the anti-m4a, anti-m2, and anti-m1a antibodies on the ability of G_o to restore high affinity agonist binding to the purified mAChR were evaluated (Fig. 1, C–E). Preincubation of the receptors with anti-m4a (Fig. 1C) antibodies totally prevented the G_o-induced high affinity state of the receptor, and only the low affinity state was observed (Table 1). The anti-m2 antibodies had a somewhat smaller effect (Fig. 1D). These results support the suggestion that additional domains within the third cytoplasmic loop, in addition to the amino-terminal 16 or 17

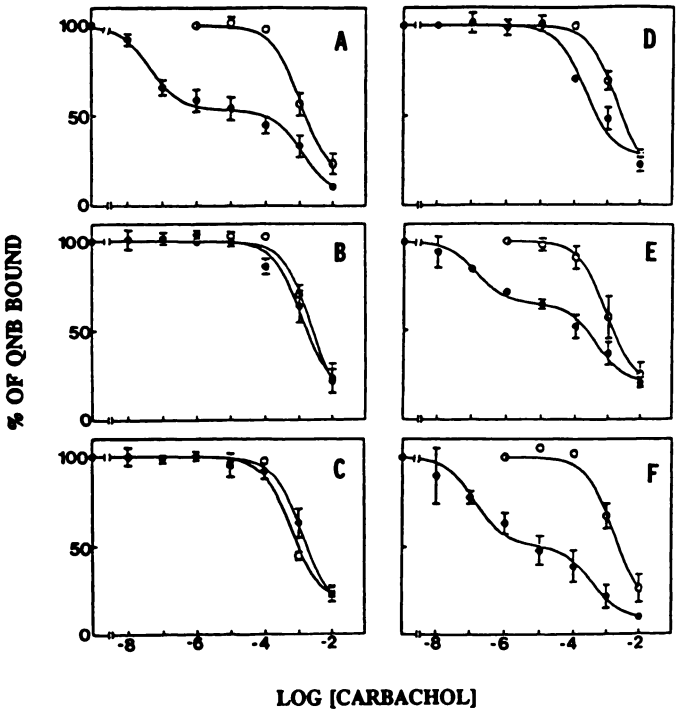

Fig. 1. Effects of different antibodies on the ability of G_o to induce high affinity agonist binding to purified and reconstituted chick heart mAChRs. Receptors were purified from chick heart and preincubated with buffer alone (A), anti-m4b antiserum (B), anti-m4a antiserum (C), anti-m2 antiserum (D), anti-m1a antiserum (E), or a representative nonimmune serum (F). The receptors were then reconstituted with or without G_o as indicated. Competitive inhibition by carbachol of [^{3}H]QNB binding to control mAChR (A), anti-m4b (B), anti-m4a (C), anti-m2 (D), or anti-m1a (E) antisera-preincubated mAChR, or a representative nonimmune serum-preincubated mAChR (F) was carried out, and the ligand binding assays were performed as described in Experimental Procedures. ○, Receptors with no G_o added; ●, receptors reconstituted with G_o. The results shown are representative of three separate experiments performed in duplicate.

TABLE 1

Binding parameters obtained from computer analysis of binding studies

The data shown in Fig. 1 were analyzed with LIGAND. K_H and K_L refer to K_i values for high and low affinity states, respectively, as determined by curve fitting with LIGAND. R_H and R_L refer to the percentage of receptors exhibiting the respective affinity. The values are mean ± standard error of three experiments performed in duplicate.

	K_H	R_H	K_L	R_L
	μM	%	μM	%
Control receptors (no antibody)				
R[a] − G_o	ND[b]	ND	100 ± 3.2	100
R + G_o	0.03 ± 0.004	50 ± 10	110 ± 2.5	50 ± 4.6
Receptors preincubated with anti-m4b antibodies				
R − G_o	ND	ND	128 ± 11	100
R + G_o	ND	ND	116 ± 18	100
Receptors preincubated with anti-m4a antibodies				
R − G_o	ND	ND	64 ± 4.4	100
R + G_o	ND	ND	203 ± 23	100
Receptors preincubated with anti-m2 antibodies				
R − G_o	ND	ND	179 ± 19	100
R + G_o	ND	ND	67 ± 3.3	100
Receptors preincubated with anti-m1a antibodies				
R − G_o	ND	ND	122 ± 3.2	100
R + G_o	0.05 ± 0.009	43 ± 7.6	189 ± 3.4	57 ± 5.5
Receptors preincubated with nonimmune serum				
R − G_o	ND	ND	151 ± 19	100
R + G_o	0.03 ± 0.008	53 ± 8	75 ± 6.2	47 ± 6

[a] R, receptor.
[b] ND, not detected.

amino acids, can participate in the coupling of mAChRs to G_o. On the other hand, the anti-m1a antibodies (Fig. 1E) against centrally located amino acid residues, as well as anti-m1b antibody (data not shown) against the amino-terminal region of the third cytoplasmic loop of the m1 mAChR, were not effective in preventing the G_o-induced high affinity state of the chick heart mAChR. These dose-response relationships were best fit to a two-state model (Table 1) and were similar to the results obtained with no antibody or with nonimmune sera.

Effects of antibodies to modulate the ability of chick heart mAChRs to stimulate GTPγS binding to G_o. To further probe the direct interaction of agonist-stimulated mAChRs with G_o, we monitored for agonist-stimulated association of GTPγS with G_o. Using the reconstituted chick heart mAChR and G_o, GTPγS binding was studied as a function of time in the presence of the agonist carbachol, with or without the antagonist atropine. The best stimulation of GTPγS binding by carbachol was obtained using 5 pmol of G_o/1 pmol of receptor, under the given experimental conditions (Fig. 2A). In agreement with previous studies (18, 20), marked effects of the agonist on GTPγS binding were observed and the effects of carbachol were prevented by the antagonist atropine. Under these conditions, specific binding due to the presence of carbachol accounted for 75% of the total binding observed. (In the presence of carbachol, after a 30-min incubation, the level of binding was ~0.17 pmol of GTPγS bound/pmol of G_o, in contrast to ~0.04 pmol of GTPγS bound/pmol of G_o in the presence of atropine.) When the reconstituted receptors were preincubated with the m4b antibody (Fig. 2B) before reconstitution with G_o, there was a near-complete inhibition of carbachol-stimulated GTPγS binding, inasmuch as specific binding was <3% of the total binding. Preincubation with the m4a (Fig. 2C) and m2 (Fig. 2D) antibodies also inhibited carbachol stimulation of GTPγS binding to 12% and 19% of that obtained in the absence of antisera (Fig. 2A). Somewhat surprisingly, the m1a antibody (Fig. 2E) also inhibited the GTPγS binding; however, the effects were significantly smaller than those obtained with the other antisera, inasmuch as specific binding was reduced to 53% of that observed in the absence of antiserum. Carbachol-stimulated GTPγS binding was not affected by preincubation of receptors with nonimmune serum (Fig. 2F); under these conditions, the specific binding was the same as with no antiserum (75% specific binding). These results suggest that interference with interaction of the amino-terminal and central regions of the third cytoplasmic loop of the chick heart mAChR with G_o can perturb the ability of the agonist-activated receptors to promote GTPγS binding to G_o. The effects of the m1 antibodies were much smaller, although not insignificant. The degree of effect appeared to be somewhat greater than the weak ability of these antisera to immunoprecipitate the chick heart mAChR (see accompanying paper, Fig. 4).

Effects of antibodies to modulate receptor-stimulated GTPase activity of G_o. To test the effects of the antibodies on a third indicator of receptor/G_o interaction, the effects of the various antibodies on the mAChR-stimulated GTPase activity of G_o were assessed (Fig. 3). Under control conditions, the addition of the agonist carbachol to mAChR/G_o vesicles markedly stimulated the GTPase activity. The stimulation due to carbachol was ~15-fold and was prevented by simultaneous exposure to atropine. The m4b antibodies, when incubated with

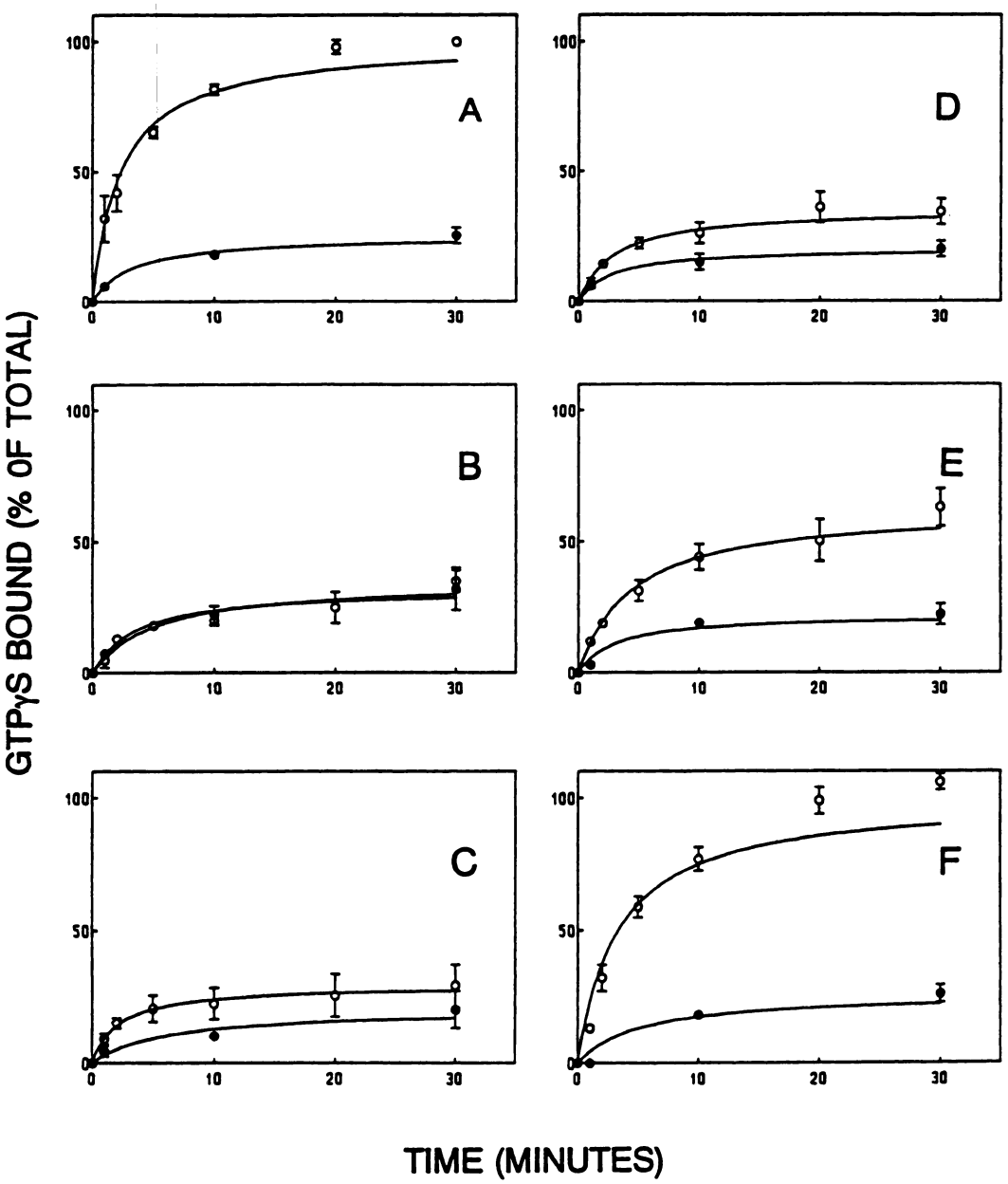

Fig. 2. Effect of different antibodies on the ability of carbachol to stimulate GTPγS binding to G_o after reconstitution of purified G_o with purified chick heart mAChR into phospholipid vesicles. Reconstituted vesicles were preincubated with buffer alone (A), anti-m4b antibody (B), anti-m4a antibody (C), anti-m2 antibody (D), anti-m1a antibody (E), or a representative nonimmune serum (F). The receptors were then reconstituted with G_o, using 5 pmol of G_o/1 pmol of purified receptor, for 1 hr on ice. GTPγS binding assays were performed as described in Experimental Procedures. ○, Receptors with 0.1 mM carbachol; ●, receptors with 0.1 mM carbachol plus 0.2 mM atropine. The data shown are representative of three separate experiments performed in duplicate.

reconstituted receptors before addition of G_o, resulted in almost total inhibition of agonist-stimulated GTPase activity; the stimulation by carbachol under these conditions was <7% of the control (Fig. 3B). These results were consistent with the finding that these antibodies also produced almost total inhibition of agonist-stimulated GTPγS binding. The m4a and m2 antibodies also inhibited the carbachol-stimulated GTPase activity (Fig. 3, C and D). With the m4a and m2 antisera the stimulations due to carbachol were only 2.2-fold and 2.7-fold, respectively, compared with the 15-fold stimulation observed under control conditions. The m1a antibodies also inhibited the carbachol-stimulated GTPase activity, but to a lesser degree than the other antibodies (Fig. 3E). With the m1a antiserum the carbachol stimulation was 9-fold, or 60% of the stimulation observed for the control. The lesser effects of the m1a antiserum (compared with the m2 and m4 antisera) paralleled the effects of this antiserum to inhibit agonist-stimulated GTPγS. However, whereas the m1a antiserum, as well as the m1b antiserum (accompanying paper, Fig. 4), have been shown to immunoprecipitate only 10–15% of ligand-binding activity of the chick heart mAChR, the comparatively larger effects of these antisera on agonist-stimulated GTPase activity and GTPγS binding may be due in part to nonspecific activities caused by the high concentration of antibodies used in these experiments. Effects of lower concentrations of antisera have not been tested. There was no inhibition of the carbachol-stimulated GTPase activity when a nonimmune serum or a control IgG was used (Fig. 3F). The data are consistent with those obtained with the GTPγS binding studies. Overall, the results provide further support for the concept that several domains in the third cytoplasmic loop of mAChRs can modulate mAChR/G_o interactions.

Effects of peptide antigens on mAChR/G_o interactions. In evaluating the aforementioned studies of functional effects of antibodies, a consideration was that some of the effects of the antibodies on receptor/G protein interactions might be due to steric hindrance caused by the binding of large antibody

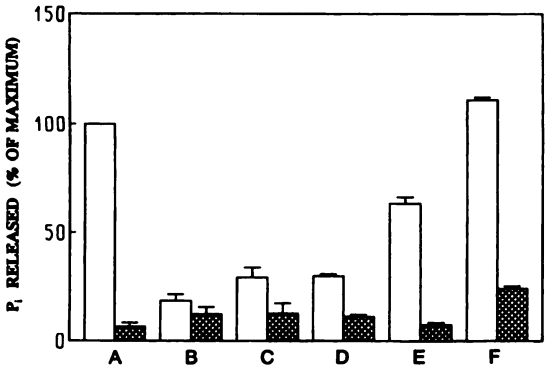

Fig. 3. Effect of different antibodies on the ability of carbachol to stimulate the GTPase activity in phospholipid vesicles containing chick heart mAChR and G_o. Reconstituted vesicles were preincubated with buffer alone (A), anti-m4b antibody (B), anti-m4a antibody (C), anti-m2 antibody (D), anti-m1a antibody (E), or a representative nonimmune serum (F). The receptors were then reconstituted with G_o, using 5 pmol of G_o/1 pmol of purified receptor, for 1 hr on ice. The assays of GTPase activity were performed as described in Experimental Procedures. The data shown are representative of three or four experiments performed in duplicate. The values shown were corrected for basal activities. The actual activities observed under control conditions were as follows: with carbachol, 0.33 mol of P_i released/mol of G_o; with atropine, 0.028 mol of P_i released/mol of G_o. □, Experiments performed in the presence of carbachol; ■, experiments performed with carbachol plus atropine.

molecules to the receptors. As one test to eliminate this possibility, we reasoned that, if the effects of the antibodies were due to specific blockade of receptor/G_o interactions, then the peptide antigens used in the preparation of the antibodies should also interfere with receptor/G_o interactions. Therefore, these peptides were tested for their ability to perturb the mAChR/G_o interactions in GTPγS binding assays. For these studies, G_o was preincubated with the indicated peptides before addition to receptor-containing vesicles. Assays then were performed to determine agonist-stimulated GTPγS binding. Under control conditions, the specific binding in the presence of carbachol was 71% of the total binding. The peptides used to produce the m4b and m4a antisera reduced the specific binding caused by carbachol to 25% and 28%, respectively, of the specific binding observed in control conditions (Fig. 4, B and C, respectively). Similarly, the m2 peptide also caused marked inhibition of agonist-stimulated GTPγS binding to G_o, to a level that was 30% of the control (Fig. 4C). The m1 peptide also reduced this activity, but to a lesser degree, such that the carbachol stimulation was 51% of that observed with no peptide (Fig. 4D). The results obtained are consistent with those obtained with the corresponding antisera. The results described above were obtained with a fixed concentration of peptides (0.1 μg/ml). In preliminary experiments, we determined the effects of other concentrations of the peptides to inhibit carbachol-stimulated GTPγS binding to chick heart mAChR. At 10 ng/ml, the m4b peptide inhibited the carbachol stimulation by 60%, and the m2 peptide inhibited the stimulation by 30%. However, at this concentration the m1 and m4a peptides were without effect. At 1 μg/ml, the m4b peptide completely inhibited the carbachol stimulation, whereas 10 μg/ml levels of the m2 and m4a peptides were required to reach a near-complete inhibition of stimulated binding. In contrast, even at 10 μg/ml the m1 peptide only inhibited binding by 60%. Although complete concentration-dependence curves have not yet been generated, these data suggest that the m4b peptide, which corresponds to sequence in the amino-terminal region of the third cytoplasmic loop of the m4 receptor, is the most potent inhibitor of receptor/G protein interaction. The m4a and m2 peptides, which correspond to more internal sequences of the third loops of the m4 and m2 receptors, respectively, appear to be at least 1 order of magnitude less potent. The m1 peptide is clearly significantly less potent, suggesting that it interacts only poorly with G_o. Taken together, these results support the conclusion that multiple domains in the third cytoplasmic loop of the chick heart mAChR can modulate interaction with G_o. However, interference with the amino-terminal region of this domain appears to have the most striking effects.

Discussion

The antipeptide antibodies prepared against the nonhomologous regions of the cytoplasmic segments between the fifth and the sixth transmembrane domains of the different mAChR subtypes exhibited different reactivities in their abilities to perturb mAChR/G_o interactions. We anticipated that antibodies against these domains would be useful in functional studies, because analogous domains have been implicated in the coupling of various G protein-coupled receptors to specific G proteins (21–24). This was borne out by our finding that several of the antibodies were capable of perturbing the interaction of chick heart mAChR with G_o. The results described herein provide the first demonstration that the amino-terminal regions as well as the central portions of the third cytoplasmic loops can play a role in the interactions of mAChR with G_o that result in high affinity agonist binding to the mAChR. In addition, the results may also suggest that these same domains are also important in the ability of these receptors to stimulate GTPγS binding and GTPase activity of G_o, although the latter effects may be a secondary expression of the lack of ternary complex formation.

The present findings with studies of purified receptors complement previous cellular studies of chimeric receptors (6, 8, 9, 25) and deletion mutants (7) that have provided strong evidence that the amino acid residues within the third cytoplasmic domain are important in defining the specificity of coupling of mAChR subtypes to their respective G proteins. Notably, the exchange of the entire third cytoplasmic domain between the m1 and m2 mAChRs caused a reversal in the abilities of the hybrid receptors to couple to specific ion channels in oocytes (6), whereas deletion of 75% of the third cytoplasmic domain of the m1 receptors, with retention of the amino- and the carboxyl-terminal ends, did not alter m1 mAChR stimulation of PI hydrolysis (7). Chimeric receptors composed of m1 mAChR and the membrane-proximal domains of the third cytoplasmic loop of the turkey β-adrenergic receptor were shown to stimulate G_s and adenylyl cyclase (8). Although these results show that substitution of the β-adrenergic receptor sequence into mAChRs allows mAChRs to signal G_s, our data show that the same region of the receptor appears to be involved in the ability of native mAChRs to complex with G_o.

Other studies have also suggested that, in addition to the amino-terminal portion of the 5–6_i loop, other cytoplasmic domains may also participate in mAChR/G protein interactions. The central portions of the 5–6_i loop have not been considered likely candidates, because of the results of studies in which 75% of the loop from m1 mAChR was deleted with no apparent loss of receptor function (7). However, the results of

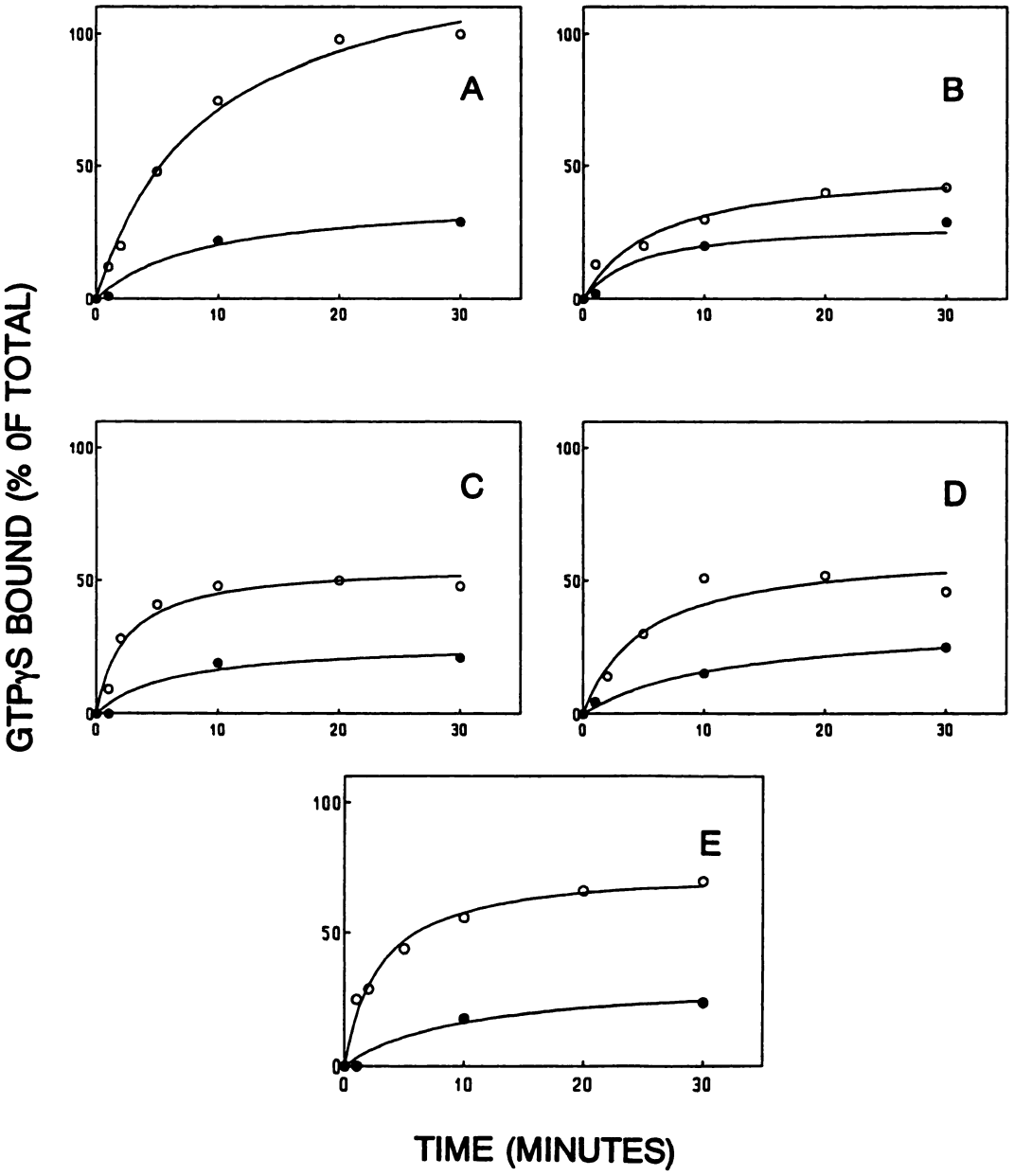

Fig. 4. Effects of different peptides used as antigens on mAChR/G_o interactions. Vesicles containing chick heart mAChRs were reconstituted with G_o alone or with G_o preincubated with different peptides (0.1 μg/ml) for 1 hr on ice, using 5 pmol of G_o/1 pmol of receptor. A, G_o preincubated with buffer alone; B, m4b peptide; C, m4a peptide; D, m2 peptide; E, m1a peptide. GTPγS binding assays were performed as described in Experimental Procedures. ○, Receptors with 0.1 mM carbachol; ●, receptors with 0.1 mM carbachol plus 0.2 mM atropine. The data shown are from a representative experiment performed in duplicate.

our studies suggest that perturbation of domains located in the central portion of the chick heart mAChR can markedly inhibit the interaction of this mAChR with G_o. The interaction of the mAChR with antibodies directed towards these domains perturbed all three aspects of receptor/G protein interaction that were tested. One possible explanation of the results is that each of these regions is important in defining receptor/G protein interactions. Another explanation is that the interaction of a bulky antibody sterically hinders access of G_o to the mAChR, even though the antibody may not directly interact with the same site on the receptor as G_o. Arguing against this possibility are the results obtained with the peptides used to prepare the antibodies. Incubation of these peptides with G_o also prevented the interaction of the chick heart mAChR with G_o. The m4b peptide made against the amino-terminal portion of the loop appears to be the most potent, but the m2 and m4a peptides also exerted significant effects. The m1 peptide was the least potent, suggesting that this sequence interacts with G_o very poorly. Taken together, the results support the concept that the amino-terminal as well as more internal portions of the third cytoplasmic loop can participate in, or modulate, mAChR/G_o interactions.

It is interesting to note that the antibodies had different effects, in quantitative terms, when they were used to interfere with the ability of G_o to restore high affinity agonist binding to the mAChR than when they were used to perturb the ability of the agonist-activated mAChR to stimulate GTPγS binding and GTPase activity of G_o. It is tempting to speculate that the amino-terminal region of the loop might be a major structural determinant on the receptors for G protein coupling but that other areas of the loop may modulate this interaction. Of particular interest to our work is the finding that phosphorylation of mAChRs can modulate mAChR/G protein interaction (17, 26–28).[1] These phosphorylation reactions involve phos-

[1] R. M. Richardson and M. M. Hosey, unpublished observations.

phorylation of serine and threonine residues (17, 29–31). The only serine residues present in any of the mAChR subtypes are located in the third cytoplasmic loops, which are quite serine-rich. Thus, it is likely that the observed effects of phosphorylation on receptor/G protein interactions involve residues of the third cytoplasmic loops. Further studies should reveal more details about these interesting interactions.

Acknowledgments

We are grateful to Mrs. J. Ptasienski for technical assistance.

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