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Subtype-Specific Antibodies for Muscarinic Cholinergic Receptors. I. Characterization Using Transfected Cells and Avian and Mammalian Cardiac Membranes

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SUMMARY

Polyclonal antibodies to a number of synthetic peptides corresponding to sequences of human muscarinic acetylcholine receptor (mAChR) subtypes m1, m2, and m4 have been prepared, in order to obtain specific tools with which to study the properties of the different receptors. Hydrophilic peptides from each subtype were chosen from the large intracellular loop between the fifth and the sixth transmembrane domains, because these loops are distinct in each of the mAChR subtypes and contain determinants for coupling to different GTP-binding proteins, as well as potential sites for phosphorylation. Five different antibodies were prepared and tested for their reactivity and specificity toward different mAChR subtypes by immunoprecipitation of ligand-binding activity and receptor protein and by immunoblot analysis. Each of the antisera immunoprecipitated its respective mAChR subtype, as evidenced by precipitation of 50–70% of the ligand-binding activity from stably transfected cells expressing the respective mAChR subtype. Very little cross-reactivity toward other subtypes was observed. We used these subtype-specific antibodies to probe the nature of the mAChR subtypes naturally expressed in avian and mammalian cardiac membranes. In tests using the antibodies with cardiac mAChRs, differences in the avian and mammalian mAChR subtypes were detected. Only the anti-m2 antibodies reacted with mAChR from porcine heart, confirming previous pharmacological and molecular biological studies that suggested that this tissue expressed only the m2 mAChR subtype. In contrast, both the anti-m2 and anti-m4 mAChR antibodies immunoprecipitated ~50% of the mAChRs solubilized from chick heart membranes. The combined or successive use of anti-m2 and anti-m4 antisera in immunoprecipitation and/or immunoblotting studies with chick heart mAChRs suggested that both antibodies appeared to recognize the same population of mAChRs from that tissue. Taken together, the results show that the subtype-specific antibodies developed in this work are useful in recognizing mAChR subtypes in cells and tissues. These antibodies should be valuable tools for further study of the properties and regulation of mAChR subtypes in various preparations. Evidence that the antibodies can modify mAChR/GTP-binding protein interactions is presented in the accompanying paper.

mAChRs mediate many of the actions of acetylcholine in neuronal and other excitable cells (1). Activation of mAChR results in coupling of the receptors to several effector systems, including inhibition of cAMP formation (2, 3), stimulation of cGMP accumulation (4), activation of phospholipase C, A2, and D (5–7), and modification of several ion channels (8–11).

mAChRs belong to the large family of G protein-coupled receptors and thus possess topological and amino acid similarities to other members of this family. Originally, mAChRs were classified as either M1 or M2, based on whether they exhibited high or low affinity for the antagonist pirenzepine (12). The recent cloning of at least five different mAChR cDNAs (13–19) has indicated that the complexity of mAChRs is more extensive than was previously thought. The five cloned mAChR subtypes are referred to as m1 through m5 (20). The odd-numbered receptors preferentially couple to stimulation of phospholipase C, whereas the even-numbered receptors act to inhibit adenylyl cyclase, in addition to weakly stimulating phospholipase C (6, 21–23).

The study of mAChR subtypes is complicated by the documented or suspected heterogeneity of mAChR subtypes present in many cells. Standard biochemical procedures using affinity labeling and/or purification are not sufficient for discriminating among the various receptor subtypes (24, 25), because most subtypes migrate on SDS gels as proteins of about 70–80 kDa. The availability of selective antibodies that could be used to specifically study the various mAChR subtypes would aid in such studies. Previous studies (26) have led to the isolation and characterization of three monoclonal antibodies against porcine

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ABBREVIATIONS: mAChR, muscarinic acetylcholine receptor; QNB, quinuclidinyl benzilate; KLH, keyhole limpet hemocyanin; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; G protein, GTP-binding protein.

cardiac mAChR. These antibodies exhibited specificity for the mammalian cardiac receptor subtype, as revealed by immunoprecipitation and immunoblotting analysis. However, these antibodies did not show any cross-reactivity with chick heart or other mAChR. Antibodies to synthetic peptides corresponding to a carboxyl-terminal sequence of mAChR subtypes were obtained by Luthin *et al.* (27) and were used to distinguish between mAChR binding sites in brain and heart by immunoprecipitation protocols. However, these antibodies were not useful for immunoblotting. Yet another polyclonal anti-synthetic peptide antiserum directed against a known mAChR-specific sequence was able to immunoprecipitate the porcine cardiac mAChR (16), but its utility has not been further described.

We have developed a series of polyclonal anti-synthetic peptide antibodies directed against specific regions of different mAChR subtypes. We chose to prepare antibodies against hydrophilic peptides from the large intracellular loop between transmembrane segments 5 and 6 (5–6_i loop) of each subtype because (i) hydrophilic domains are likely to be more exposed than the internal hydrophobic residues and antipeptide antibodies against such regions are more likely to react with the native protein (28); (ii) the 5–6_i loops are distinct in each of the mAChR subtypes, thus providing regions that have a high likelihood for successful production of subtype-selective antibodies; (iii) the 5–6_i loops are likely to contain sites for regulation of mAChRs by phosphorylation; and (iv) the 5–6_i loops contain determinants for coupling of different subtypes to different G proteins and effectors (29–32). In this communication, we report on the development and use of these antibodies to characterize specific receptor subtypes in cells and tissues. In the accompanying paper, we demonstrate the utility of these antibodies to modify receptor/G protein interactions.

Materials and Methods

Generation of antipeptide antibodies. Peptides corresponding to selected sequences of human mAChR subtypes (15) were synthesized (Multiple Peptide Systems, La Jolla, CA) with cysteine amides added to the carboxyl terminal, in order to facilitate coupling to the carrier. The peptides were coupled to KLH, according to the procedure of Green *et al.* (33). Each of the synthetic peptides was repurified by reverse phase high performance liquid chromatography before use. After coupling to KLH, the conjugates were dialyzed extensively against phosphate-buffered saline and then stored at −20°. The coupling efficiency varied between 20 and 35%.

For immunization, peptide-KLH conjugates (1 mg/ml) in phosphate-buffered saline were emulsified using a RIBI (Hamilton, MT) adjuvant system (MPL plus TDM plus CWS emulsion). The emulsion was administered to New Zealand white rabbits as follows: 0.2 ml into the popliteal lymph nodes (0.1 ml in each node), 0.2 ml intradermally (0.05 ml in each of four sites), 0.4 ml intramuscularly (0.2 ml into each hind leg), 0.1 ml intraperitoneally, and 0.1 ml subcutaneously (neck region). The rabbits were boosted 21 days after the first immunization, and subsequent boosters were given every 21 days. The administration of the antigen into the popliteal lymph node was omitted during the booster injections. The rabbits were bled 10–14 days after each boost, and the IgG was purified from the antiserum by DEAE-Affi-Gel Blue chromatography, using a protocol supplied by the manufacturer (Bio-Rad).

ELISA of rabbit immune sera using mAChR-derived synthetic peptides as immunosorbants. ELISAs were performed essentially as described by Maelicke *et al.* (34), using microtiter plates containing the mAChR peptides (1.5 μg/well in 0.125% glutaraldehyde) as immunosorbents.

Preparation of membranes. Homogenates (1/10 dilution) of porcine atria, chick heart, transfected Chinese hamster ovary cells expressing m2 or m3 mAChR, and transfected human embryonic kidney cells expressing m1 or m4 mAChR subtypes were prepared with a Polytron (Brinkman) device in 0.25 M sucrose, 20 mM potassium phosphate, pH 7.4, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1 mM iodoacetamide, 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml soybean trypsin inhibitor, 2 μg/ml leupeptin, 1 μM pepstatin, 1 μg/ml aprotinin. Crude porcine atrial and chick heart membranes (P′) enriched in mAChRs were prepared as previously described (36). Homogenates from the cultured cells were centrifuged at 800 × *g* for 10 min at 4°, to remove the nuclear debris and unbroken cells. The resultant supernatants were centrifuged at 20,000 × *g* for 40 min at 4°, to obtain the crude membrane fraction. Rat brain synaptosomal membranes were prepared according to the method of Jones and Matus (35).

Immunoprecipitation assays. Membranes (200–300 μg of protein/ml) in 10 mM potassium phosphate, pH 7.0, with protease inhibitors (1 mM iodoacetamide, 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml soybean trypsin inhibitor, 2 μg/ml leupeptin, 1 μM pepstatin, and 1 μg/ml aprotinin), were prelabeled with 1–2 nM [^{3}H]QNB at 37° for 75 min. Nonspecific binding was defined with 1 μM atropine. After labeling, the membranes were centrifuged to remove free ligand. Pellets were washed and resuspended in the phosphate buffer, and the mAChRs were solubilized at 4° for 30 min in the presence of digitonin/cholate, at a final concentration of 0.4% digitonin/0.1% cholate. After centrifugation, the indicated amounts of antipeptide antisera were added to the solubilized receptors, and the samples were rocked overnight at 4° in buffer A (20 mM HEPES, pH 7.5, 200 mM NaCl, 5 mM EDTA, 10% glycerol), such that the final concentration of digitonin was 0.08%. Aliquots (1 ml) of Immunobeads (Bio-Rad) reconstituted in buffer A were added, and the reactions were incubated for another 2 hr at 4°. The mixtures were centrifuged, and the amounts of specifically bound [^{3}H]QNB remaining in the supernatants were analyzed after filtration onto GF/A filters presoaked in 0.3% polyethylenimine. Free QNB was eliminated by the filtration step and did not contribute to the determinations. Nonspecific effects of antisera were defined by using either preimmune serum or antiserum that had been preadsorbed with the synthetic peptide (50 μg/ml) that was used as the antigen.

Immunoblotting. Several different methods for detecting receptor protein after immunoblotting were used. In general, the detection of mAChRs with standard colorimetric procedures gave less than acceptable results, due to high nonspecific staining. The immunoblotting technique that yielded the best results was one in which the receptors were immunoprecipitated with anti-mAChR antibodies bound to Protein A-agarose (Pierce) and were then probed by immunoblotting with the same antibody, followed by detection with ^{125}I-Protein A. Aliquots of Protein A-agarose were washed with 10 mM Tris·HCl, pH 7.5, incubated with the indicated dilutions of the peptide-specific antisera overnight at 4°, and then washed three times with buffer B (25 mM Tris·HCl, pH 7.5, 200 mM NaCl, 5 mM EDTA, 0.08% digitonin, 0.1% Triton X-100, 0.1% bovine serum albumin). The Protein A-agarose-bound antibodies were then incubated at 4° for 2–16 hr with digitonin-solubilized membrane extracts of the various preparations. The immunoprecipitates were washed three times with buffer B and three times with buffer B without bovine serum albumin, suspended in SDS sample buffer (37) containing 8 M urea, and subjected to electrophoresis on SDS-urea gels containing 8% polyacrylamide, followed by electroblotting onto nitrocellulose. The blots were then incubated with the indicated dilutions of antisera, in 5% bovine serum albumin in Tris-buffered saline, overnight at 4°. After standard washing and blocking procedures, the blots were incubated with ^{125}I-Protein A for 2 hr at room temperature, washed with 0.2% Tween-20 in Tris-buffered saline, dried, and subjected to autoradiography at −70°.

Results

The amino acid sequences of the peptides used in this study are shown in Table 1 and correspond to sequences of human

mAChR subtypes described by Peralta *et al.* (15). The nomenclature for designating subtypes of mAChRs is that of Bonner (20), where m1, m2, and m4 refer to the same subtypes designated as HM1, HM2, and HM3, respectively, by Peralta *et al.* (15). (We have not yet attempted to raise anti-m3 antibodies.) The titers of the antibodies were determined by ELISA, using peptides as immunosorbents (Fig. 1), and were found to vary from peptide to peptide. The highest titers were obtained with m4a peptide. The data presented in the figure were obtained with antisera obtained after the fifth boost. However, the titers increased with subsequent booster injections. In separate experiments, each antiserum was tested for its nonspecific cross-reactivity with the other peptides, and it was observed that the antisera exhibited only weak cross-reactivity with the other peptides (approximately 10–20% at lower dilutions) (data not shown). Hence, in subsequent experiments appropriate dilutions of various antisera were used. The dilutions at which various antipeptide antisera showed no nonspecific cross-reactivity were 1/1000 (m1a), 1/1000 (m1b), 1/1200 (m2), 1/2000 (m4a), and 1/1600 (m4b).

TABLE 1

Amino acid sequences of the synthetic peptides used to generate the subtype-specific antipeptide antisera

Subtype-specific antisera code	Residues	Amino acid sequence
m1a	280–293	E D E G S M E S L T S S E G C-amide
m1b	210–223	R I Y R E T E N R A R E L A C-amide
m2	277–290	G E E K E S S N D S T S V S C-amide
m4a	289–302	D K D T S N E S S S G S A T C-amide
m4b	218–231	H I S L A S R S R V H K H R C-amide

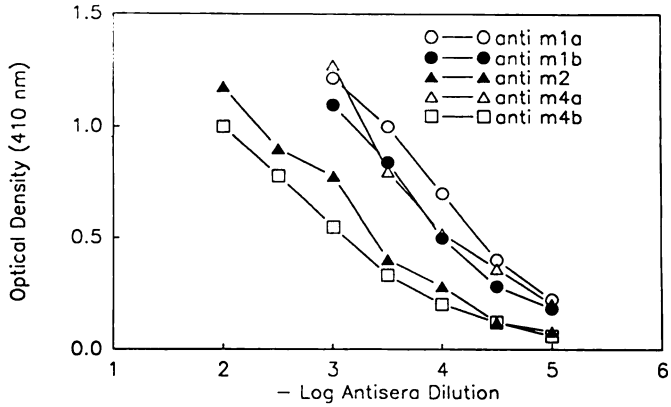

Fig. 1. ELISA of rabbit immune sera, using mAChR-derived synthetic peptides as immunosorbents. Increasing dilutions of antipeptide m1a (○), m1b (●), m2 (▲), m4a (△), and m4b (□) antisera were tested on their respective synthetic peptides as described in Materials and Methods. The values obtained from the antisera that had been preadsorbed with peptides (50 μg/ml) were subtracted from those of the antipeptide antisera.

Immunoprecipitation of mAChR subtypes from transfected cells expressing m1, m2, and m4 mAChRs. The immunoprecipitation of various mAChR subtypes, expressed in either Chinese hamster ovary cells transfected with cDNA for the m2 mAChR or human embryonic kidney cells transfected with the cDNAs for the m1 or m4 mAChR, was tested in ligand binding assays using the antisera against each subtype individually (Fig. 2). Digitonin-solubilized extracts of membranes from cells expressing the m1 mAChR were used for immunoprecipitation by m1b, m2, and m4b antisera (Fig. 2A). It was observed that the m1b antiserum immunoprecipitated

TABLE 2

Sequence alignment of different amino acid residues from various mAChR subtypes to indicate homologies

The amino acid homologies between the two sets of sequences compared to examples 1, 2, 3, and 4 are 78%, 71%, 64%, and 78%, respectively. The residues that are different in each pair are shown in **boldface**. h and c preceding the subtype numbers refer to human and chick, respectively.

Example	mAChR subtype/peptide	Residues	Amino acid sequence
1	hm1/m1b	210–223	R I Y **R** E T E N **R A** R E L A
	hm3	253–266	R I Y **K** E T E **K R T K** E L A
2	hm2/m2	277–290	G E E K E **S S** N D S **T S** V S
	cm4	297–310	**Q** E E K E **T** S N **E S S T** V S
3	hm4/m4a	289–302	D K D T S N E S S **S G** S A T
	cm4	299–312	E K E T S N E S S **T** V S **M** T
4	hm4/m4b	218–231	H I S L A S R S R V **H K** H **R**
	cm4	227–240	H I S L A S R S R V **R R** H K

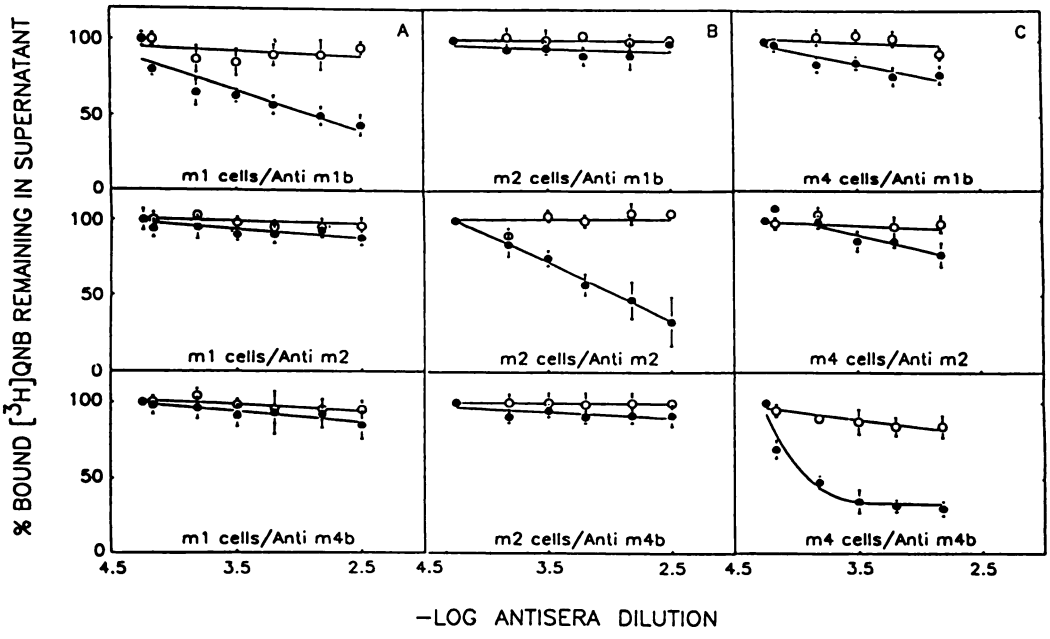

Fig. 2. Immunoprecipitation of mAChRs from cells expressing m1, m2, and m4 mAChRs. Receptors were prelabeled with the muscarinic antagonist [^{3}H]QNB and subsequently solubilized in 0.4% digitonin/0.1% cholate. The indicated amounts of antipeptide antisera (●) or antisera preadsorbed with the respective peptides (50 μg/ml) (○) were added to the solubilized receptors (180–200 μg of protein), and immunoprecipitation was performed as described in Materials and Methods. A, Immunoprecipitation of m1 mAChR by anti-m1b (*top*), anti-m2 (*middle*), and anti-m4b (*bottom*) antisera. B, Immunoprecipitation of m2 mAChR by anti-m1b, anti-m2, and anti-m4b antisera. C, Immunoprecipitation of m4 mAChR by anti-m1b, anti-m2, and anti-m4b antisera.

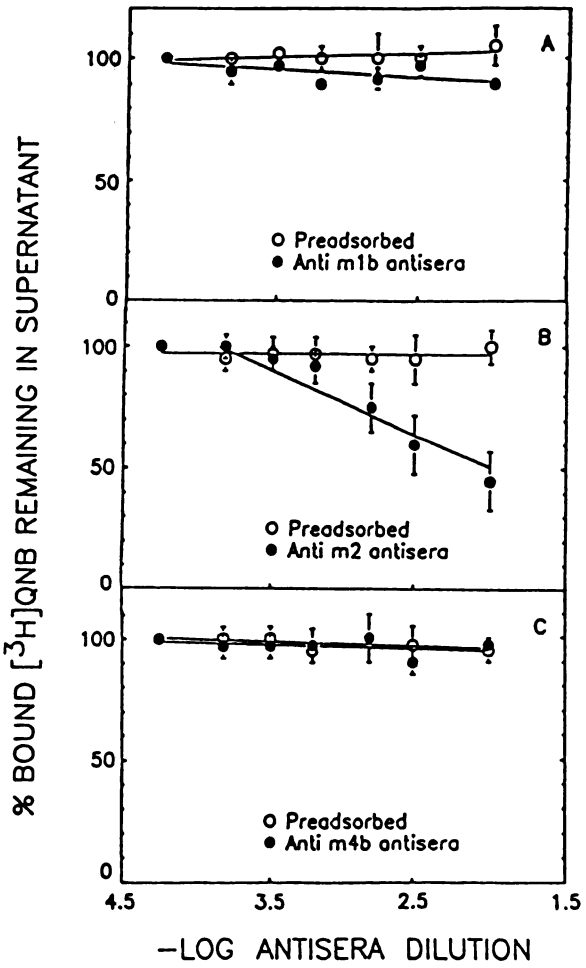

Fig. 3. Immunoprecipitation of mAChRs solubilized from porcine atrial membranes. Experiments were performed as described in the legend to Fig. 2. Before the addition of antipeptide antisera (●) or peptide (50 μg/ml)-preadsorbed antisera (○), the solubilized receptors (400–500 μg of protein) were diluted to 0.08% digitonin in buffer. A, B, and C, Immunoprecipitation of porcine atrial mAChRs by anti-m1b, anti-m2, and anti-m4b antisera, respectively.

m1 mAChR in a concentration-dependent manner, with maximum immunoprecipitation under these conditions of about 50%. In contrast, the m2 antiserum did not have any significant reactivity towards the m1 receptors, whereas the m4b antiserum immunoprecipitated <5–10% of m1 mAChR at the highest concentrations tested. Similar experiments were performed with extracts of cells expressing m2 and m4 receptors (Fig. 2, B and C, respectively). The m2 and m4b antisera specifically immunoprecipitated 65–70% and 55–60%, respectively, of the receptors against which they were directed. The cross-reactivity of the m2 antiserum with the m1 receptors was negligible, whereas the cross-reactivity with the m4 receptors was about 15% at high concentrations. The cross-reactivity of the m4 antiserum with the m1 and m2 receptors was not significantly different from that of the preimmune or preabsorbed antisera. These data show that the m1, m2, and m4 antisera immunoprecipitated the ligand-binding activity of mAChRs from stably transfected cell lines expressing m1, m2, and m4 mAChRs, respectively, with at most 5–15% nonspecific cross-reactivity. Similar results were obtained with anti-m1a and anti-m4a antisera (data not shown).

Immunoprecipitation of mAChRs solubilized from porcine atria. In view of the specificity of precipitation achieved with the expressed receptor subtypes, the mAChR subtype-specific antisera were used to characterize the mAChR subtypes in mammalian and avian heart membranes. It has been suggested that porcine atria contain only the m2 mAChR subtype, because Northern blot analysis of the mRNA indicated only the presence of the m2 mAChR (16). To further demonstrate the existence of the m2 protein product and to test the specificity of the mAChR antibodies with this tissue, solubilized membranes from porcine atria were subjected to immunoprecipitation with the m1b, m2, and m4b antisera. The results indicated that only the anti-m2 antiserum immunoprecipitated the mAChR from the solubilized porcine atrial preparation (Fig. 3). The m1 and the m4 antisera did not immunoprecipitate these mAChRs. The results are in agreement with the suggestion that only m2 mAChRs are detectable in porcine heart (16), and they demonstrate the selectivity of the antisera towards mammalian m2 receptors.

Immunoprecipitation of mAChRs solubilized from chick heart. Previous studies have demonstrated that chick heart mAChRs are pharmacologically and structurally distinct from their mammalian counterparts (38, 39). Recent cDNA cloning studies based on screening of a genomic chicken library have led to the identification of an avian m4 receptor that appears to be expressed in chick heart (40). However, other studies have suggested that additional mAChR subtypes may also be expressed in chick heart.[1] The mAChR antisera were, therefore, tested to characterize the nature of the mAChR in solubilized chick heart membranes (Fig. 4). The antibodies directed against m1 mAChR only weakly recognized the chick heart mAChR (Fig. 4A); however, these antibodies appeared to give significant immunoprecipitation of a small percentage of QNB-binding activity at high concentration of antibodies. In contrast, the m2 and the m4b antisera immunoprecipitated about 50–55% and 40–50% of the mAChRs, respectively (Fig. 4, B and C). These observations suggested that m2 and m4 subtypes may be present in chick heart. To further test this possibility, we used m2 and m4b antisera together to immunoprecipitate the mAChRs from solubilized chick heart preparations, to determine whether additive immunoprecipitation could be achieved. No additive effects were observed at any concentration of antisera. Immunoprecipitation occurred to almost the same extent as that with the m2 antiserum alone (Fig. 4D). This result suggested that the m2 and m4b antisera may have immunoprecipitated the same population of mAChRs from the chick heart. To further test this, first the chick heart mAChRs were immunoprecipitated with either the m2, m4a, or m4b antisera and then the precipitated proteins were subjected to immunoblotting analysis with each of the three antibodies (separately), followed by detection with ^{125}I-Protein A. In all cases, the protein precipitated with one antibody was recognized equally well on the Western blots by the other antibodies (Fig. 5). As a positive control, we probed the authentic human m2 and m4 receptors from stably transfected cells under the same conditions. In these cases, the antibodies only exhibited reactivity towards their respective receptors (Fig. 5). (The m4a antibody appeared to be slightly less effective at immunoprecipitation than the other two antibodies.) These results strongly

[1] K. Wortley, and M. Hosey. Unpublished observation.

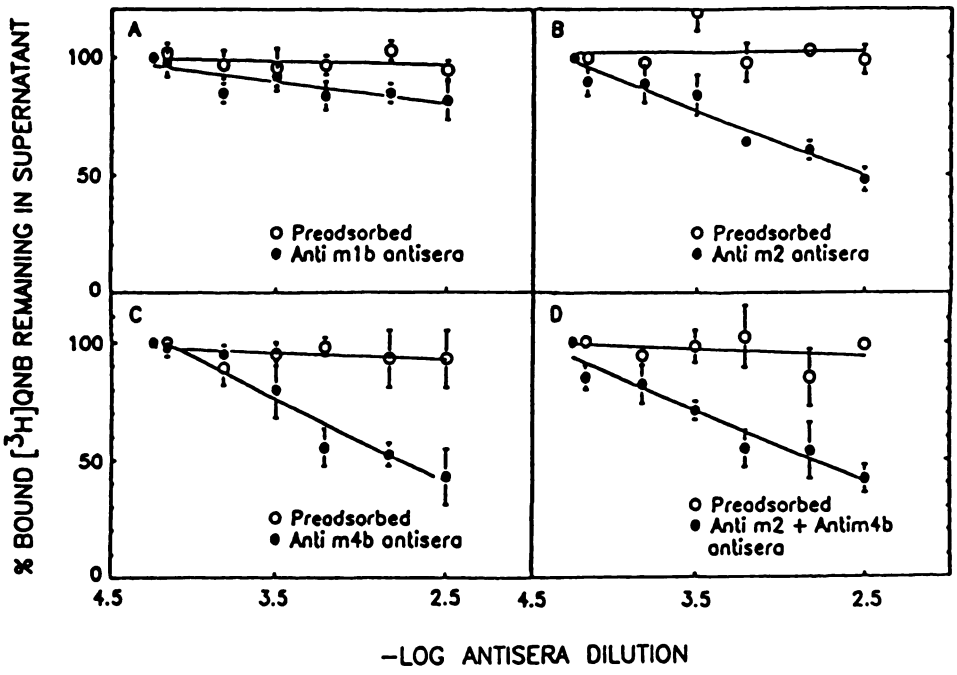

Fig. 4. Immunoprecipitation of mAChRs solubilized from chick heart membranes. Chick heart membranes were prelabeled with the muscarinic antagonist [^{3}H]QNB, and mAChRs were immunoprecipitated as described in the text. A, B, and C, Immunoprecipitation of chick heart mAChRs by anti-m1b, anti-m2, and anti-m4b antisera, respectively. D, Immunoprecipitation of chick heart mAChRs in the presence of equal amounts of anti-m2 and anti-m4b antisera.

suggested that the m2 and m4b antisera both recognized the same population of chick heart mAChRs.

Western blot analysis of the immunoprecipitates of mAChRs from rat brain, porcine atria, and chick heart. The sizes of the protein products of the m1, m2, and m4 genes are predicted to be slightly different (15, 17), and the contribution of glycosylation to the apparent size of each protein is not known. As an examination of the sizes of the various proteins corresponding to the m1, m2, and m4 mAChR subtypes, we visualized the immunoprecipitated mAChR proteins from the various sources after immunoblot analysis with ^{125}I-Protein A. These experiments were performed with dilutions of the antisera at which there should be minimal cross-reactivity (Fig. 2). The m1a and the m1b antisera (the m1b antiserum recognizes m1 and m3 receptors; see below) recognized protein(s) of about 77 kDa from rat brain membranes (Fig. 6, A and B), but very little or no reactivity was observed with mAChR from porcine and chick heart. (A very faint, almost not detectable, band of 77 kDa was detected in the chick heart sample with m1b antiserum.) The m2 antiserum detected proteins of approximately 77 kDa from rat brain, porcine atria, and chick heart (Fig. 6C). The m4a and m4b antisera each detected proteins of 77 kDa from rat brain and from chick heart (Fig. 6, D and E), but no reactivity was detected with extracts from porcine atria.

In similar experiments, Western blot analyses of the immunoprecipitates of mAChRs from transfected cells expressing the mAChR subtypes were performed, and the data showed that the m1a, m2, m4a, and m4b antisera each specifically detected respective mAChR subtypes of ~77 kDa. The m1b antiserum, in addition to detecting m1 mAChR, also showed cross-reactivity with the m3 mAChR subtype (data not shown). Although we have not prepared m3 antiserum, we predicted the observed cross-reactivity of the anti-m1b antiserum with m3 mAChR, based on the sequence homologies shown in Table 2, example 1.

We also used the antibodies to immunoprecipitate [^{3}H]propylbenzilylcholine mustard-labeled mAChRs from cells expressing mAChR subtypes and from chick heart membranes (data not shown). In all cases, specific immunoprecipitation of affinity-labeled receptors was obtained, and the results were in good agreement with those of the immunoblotting experiments. Taken together, these results demonstrate that the apparent sizes of the protein products of m1, m2, and m4 genes were similar in the various cells and tissues analyzed.

Discussion

This work presents results concerning the development, characterization, and use of antipeptide antibodies that exhibit a high degree of selectivity for various subtypes of mAChRs. The major use of the mAChR subtype-selective antibodies in the present study was to characterize the subtypes of mAChR proteins that are expressed in mammalian and avian heart. The antipeptide antibodies were useful in immunoprecipitation and immunoblotting studies. Although previous studies have documented the development and use of different types of antisera towards mAChRs (26, 27), the data from the present studies using cells transfected with mAChR cDNAs demonstrated the selectivity of the various antibodies in recognizing the mAChR subtypes and provided the basis for using the antibodies to characterize the subtypes in chick heart.

A goal of the studies presented here was to determine the characteristics of the subtype(s) of mAChR present in chick heart. Pharmacological and structural analyses have indicated that chick heart mAChRs are different from mammalian heart mAChRs (38, 39). The results of Northern blot analysis (40) suggest that mRNA for an avian m4 receptor is present in chick heart; however, other mAChR subtypes might also be expressed in this tissue. We found that the anti-m2, anti-m4a, and anti-m4b antisera each precipitated what appeared to be a common receptor pool from chick heart. After aligning residues 277–290 from the human m2 mAChR (used as the anti-m2 antigen) with residues 297–310 from the avian m4 mAChR (cm4) recently reported by Tietje *et al.* (40), we observed that

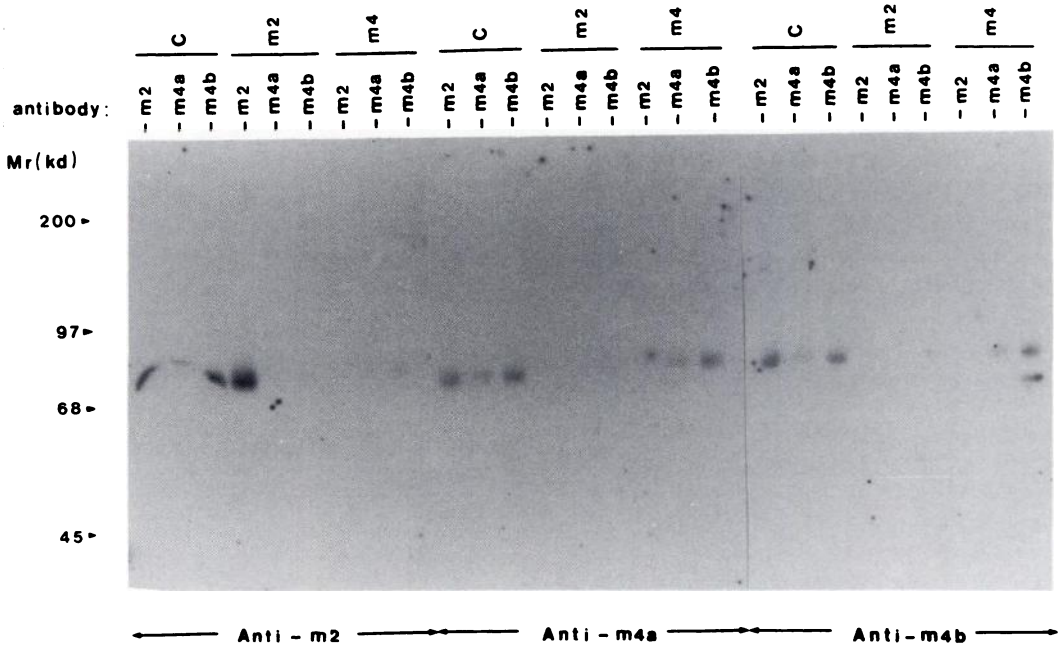

Fig. 5. Cross-reactivities of mAChRs from chick heart with anti-m2 and anti-m4b antibodies. Solubilized receptors were first immunoprecipitated with the antibodies indicated in the *top row*, by incubation of the solubilized extracts for 2 hr at 4° with 50 μl of Amino Link gel (Pierce), to which the respective antibodies were covalently coupled according to the manufacturer's protocol. After incubation, the beads were washed three times with solubilization buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, leupeptin, and soybean trypsin inhibitor and were then suspended in 30 μl of SDS sample buffer, followed by electrophoresis on SDS gels. After transfer of the proteins to nitrocellulose by electroblotting, each immunoprecipitate was tested for reactivity towards other antibodies (indicated in the *bottom row*) by immunoblotting. The remaining procedures were as described in Materials and Methods. *C*, Chick heart mAChR; *m2* and *m4*, receptors from cells expressing human m2 and m4 mAChRs, respectively. The dilutions of anti-m2, anti-m4a, and anti-m4b antibodies used in these experiments were 1/1600, 1/2000, and 1/2000, respectively.

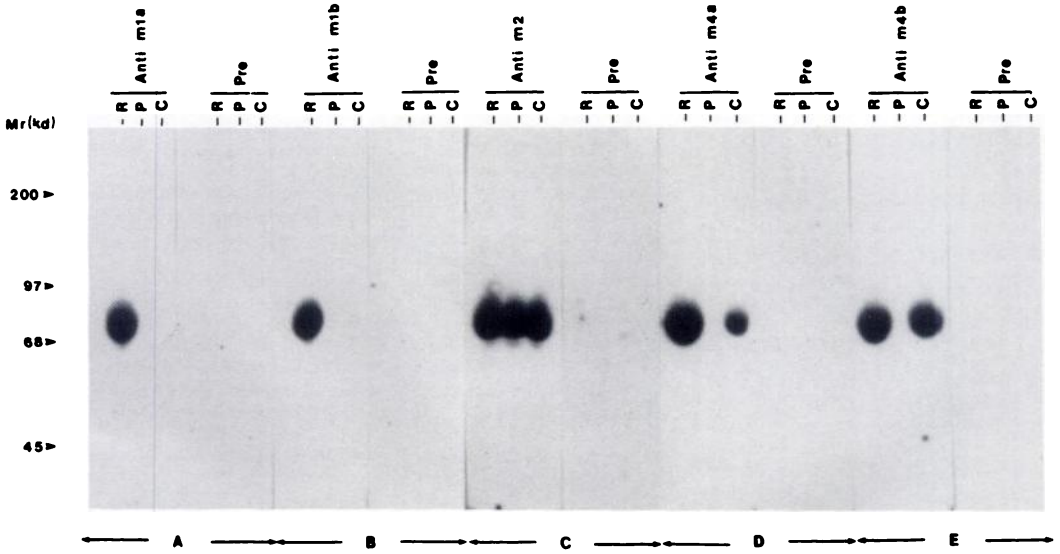

Fig. 6. Western blot analysis of the immunoprecipitates of mAChRs from rat brain, porcine atria, and chick heart. The experiments were performed as described in Materials and Methods. A was probed with 1/1600 dilution of anti-m1a antiserum, B with 1/1000 dilution of anti-m1b antiserum, C with 1/1200 dilution of anti-m2 antiserum, D with 1/2000 dilution of anti-m4a antiserum, and E with 1/1600 dilution of anti-m4b antiserum. *Pre*, peptide (50 μg/ml)-preadsorbed antisera, with the same dilution as that of the antisera in each case. *R*, *P*, and *C* as indicated in the *top row*, mAChRs from rat brain, porcine heart, and chick heart, respectively.

the sequences contain 9 of 14 identical amino acids and several other conservative substitutions (Table 2, example 2). Thus, it is conceivable that the anti-human m2 antibodies may have recognized the avian m4 receptors. However, the sequence of the avian m2 mAChR that corresponds to that used to prepare the anti-human m2 antiserum is even more highly conserved,[1] with only one amino acid that is different. The relevant sequences of the chick m4 receptor (40) are highly similar to the peptides used to prepare the anti-m4a and anti-m4b antibodies (Table 2, examples 3 and 4). In view of these considerations, and because our data show that the anti-human m2 and anti-human m4 antibodies both recognized chick heart mAChRs, we cannot say whether avian m4 or m2 receptors were recognized. A possibility is that there may be m2 and m4 receptors in chick heart that are recognized by both the anti-human m2 and m4 antibodies. The affinities of mAChRs for agonists, as determined in *in vitro* [^{3}H]QNB competition experiments, differ during development of the chick heart (54). Hence, it is conceivable that there might exist a temporal program for selective mAChR subtype(s) expression in chick heart. This will be ascertained in a future developmental study, using the mAChR subtype-specific antibodies described in this work and Northern blot analysis of mAChR subtype mRNA.

The antipeptide antibodies prepared against the nonhomologous region of the cytoplasmic segments between the fifth and the sixth transmembrane domains of the different mAChR subtypes exhibited high selectivity towards mAChR subtypes. We opted to raise the antipeptide antibodies against portions of this intracellular segment from different mAChR subtypes because these cytoplasmic segments display the least sequence similarity among the different mAChR subtypes and other G protein-coupled receptors. However, peptide m1b and peptide m4b have sequence similarities with stretches of the m3 and m2 receptors, respectively. Both of these peptides are from sequences in the amino-terminal domains of the third cytoplasmic loops, in an area that has been strongly implicated in receptor/G protein interactions (41–43). Thus, we also anticipated that antibodies against these domains would be useful in various types of functional studies. First, because analogous domains have been implicated in the coupling of various G protein-coupled receptors to specific G proteins (41–43), we envisioned that antibodies against these domains might be useful in defining receptor/G protein interactions. This is demonstrated by the results presented in the accompanying paper.

A second anticipated use of the antibodies is for the study of processes that regulate signal transduction through the mAChR subtypes. As is the case with other members of the G protein-coupled receptor family, mAChRs desensitize and down-regulate in response to prolonged activation by agonists. The molecular basis of these events is not known with certainty, but phosphorylation of the receptors has been implicated to play a role in both of these processes (6, 44–46). Results from our laboratory have previously demonstrated that agonists induce phosphorylation of mAChRs on serine and threonine residues in intact chick and porcine cardiac cells (38, 46, 47). In addition, mAChRs have been shown to be substrates for phosphorylation *in vitro* (48–52) by several protein kinases. Because the only available serine and threonine residues are located in the $5–6_i$ loops, the antibodies could also be useful in future studies of the role of receptor phosphorylation.

A third potential use of the antibodies may be to ascertain what other membrane or cytoplasmic protein components are associated with various mAChR subtypes. Of interest is the recent finding that *ras* and GAP (GTPase-activating protein) appear to interfere with mAChR activation of K^+ channels (53). The antireceptor antibodies may be useful to determine whether the activated mAChRs associate with GAP and/or other potential target proteins. Further studies will address these issues.

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