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Antibodies to a Synthetic Peptide Can Be Used to Distinguish between Muscarinic Acetylcholine Receptor Binding Sites in Brain and Heart

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SUMMARY

Since the reports elucidating the sequence of four subtypes of muscarinic cholinergic receptors appeared, it has been clear that pharmacological approaches to the study of subtypes of these receptors are inadequate to selectively detect one subtype in the presence of the others. One methodology that can provide more selective reagents with which to study these subtypes is immunology. Thus, using the information on the primary sequence of these receptors available in the literature, rabbits were injected with an oligopeptide, CRKIPKRPGSVHRTPSRQ, conjugated to keyhole limpet hemocyanin. This oligopeptide (m_1 C-terminal peptide) corresponds to the 17-amino acid sequence of the carboxyl terminus of a rat m_1 muscarinic receptor. This portion of the amino acid sequence of the muscarinic receptor protein has been shown to be unique to the m_1 receptor and has not been found in the other subtypes of the receptor thus far sequenced. The antisera (anti-m_1 antisera) had high titer against the m_1 C-terminal peptide in a solid phase radioimmunoassay. The anti-m_1 antisera were shown to immunoprecipitate [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding activity solubilized from rat forebrain. [^{3}H]Pirenzepine ([^{3}H]PZ) has been shown to interact with a subset of [^{3}H]QNB binding sites in forebrain and heart. The anti-m_1 antisera were shown to immunoprecipitate [^{3}H]PZ binding sites in cerebral cortex, hippocampus, and corpus striatum, areas believed to be rich in the m_1 subtype of the muscarinic receptor. Although [^{3}H]PZ binding activity was present in receptor preparations solubilized from heart, neither [^{3}H]PZ- nor [^{3}H]QNB-binding activities could be immunoprecipitated from this tissue using the anti-m_1 antisera. A monoclonal antibody raised against the porcine atrial muscarinic receptor was shown to immunoprecipitate both [^{3}H]PZ- and [^{3}H]QNB-binding activities solubilized from rat heart, but only [^{3}H]QNB-binding activity could be immunoprecipitated from forebrain using this antibody. Immunoprecipitation of [^{3}H]PZ- and [^{3}H]QNB-binding activity by anti-m_1 antisera could be inhibited by the m_1 C-terminal peptide. Peptides corresponding to the C-terminal portions of the rat m_3 and m_4 muscarinic receptor were not inhibitory in the immunoprecipitation assay. This study provides further evidence for subtypes of muscarinic receptors in rat tissues and supports the hypothesis that receptor subtypes defined using PZ can be further subclassified on the basis of differences in primary structure. The data indicate that [^{3}H]PZ binds not only to the m_1 muscarinic receptor but also to other muscarinic receptor subtypes in solubilized preparations from rat brain and heart. Therefore, [^{3}H]PZ binding cannot be assumed to measure m_1 receptors exclusively in rat tissues. Additionally, the results obtained in this study indicate that the approach of developing antibodies specifically directed toward a single subtype of muscarinic receptor, by using peptides that are uniquely represented in a given subtype, will be fruitful.

Subtypes of muscarinic acetylcholine receptors have been identified on the basis of the unique pharmacological profiles of the drugs PZ and, more recently, AF-DX116 (1-3). Goyal and Rattan (4) initially designated subtypes of muscarinic receptors as M_1 and M_2, representing receptors with high and low affinity for PZ, respectively. This classification has been supported by other functional and radioligand binding studies (1-3, 5, 6). In radioligand binding studies, both PZ and AF-DX116 have been shown to interact with high and low affinity binding sites, depending on the tissue studied. Thus, PZ interacted with high affinity sites (K_d = 10 nM) and low affinity sites (K_d = 0.3-1 μM) in forebrain whereas in such tissues as heart and cerebellum essentially all of the sites have low affinity for PZ (1). In direct binding studies [^{3}H]PZ can be used to label high but not low affinity binding sites in forebrain and select other tissues (7-9). Recent pharmacological data have led to the suggestion that M_2 receptors should be further subclassified

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ABBREVIATIONS: PZ, pirenzepine; QNB, quinuclidinyl benzilate; KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; TBS, Tris-buffered saline; CHO, Chinese hamster ovary; SDS, sodium dodecyl sulfate; AF-DX116, 11-[(2-[(dimethylamino)methyl]-1-piperidinyl)acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine-6-one.

(2, 10, 11). Thus, AF-DX116 has been shown to discriminate between cardiac and exocrine gland muscarinic receptors, both of which have been classified M_2 on the basis of their affinity for PZ. It therefore appears that multiple subtypes of muscarinic receptors may be expressed in mammalian tissues.

Several groups have described the purification (12–14) and molecular cloning (15–19) of muscarinic receptor subtypes. As a result, the primary structures of four subtypes of the muscarinic receptor have recently been reported. The cloned cDNAs for these proteins have been expressed in several different cell lines. The expressed proteins bound [^{3}H]QNB with high affinity (15, 16, 17, 19). For clarity, these four receptors will be referred to in this paper using the nomenclature of Bonner *et al.* (15), who suggested the use of the lower case 'm' (i.e., m_1–m_4). Two of these proteins (m_1 and m_4 muscarinic receptors) bound PZ with high affinity (K_d = 10 nM). The other two proteins (m_2 and m_3 muscarinic receptors) bound PZ with lower affinities (K_d = 1 and 0.1 μM, respectively) (15, 16). Thus, although four homologous receptors exist, they have a spectrum of affinities for PZ and AF-DX116 (15, 16). The relationship in brain and other tissues between high and low affinity binding sites for PZ and distinct receptor proteins remains uncharacterized to date. It was the purpose of the present study to use a combination of serological reagents and radioligand binding techniques to identify structurally defined subtypes of muscarinic receptors and to examine the relationship of high affinity PZ binding sites with the m_1 subtype of muscarinic receptor.

Materials and Methods

Tissue preparation. Cerebral cortex, striatum, hippocampus, cerebellum, and heart tissues were obtained from male Sprague-Dawley rats as previously described (8). All following procedures were performed at 4°. After dissection, tissues were homogenized using a Polytron homogenizer (setting 6; 10 sec) in 10 volumes of 10 mM Tris (pH 7.5), 1 mM EDTA, and centrifuged at 20,000 × *g* for 10 min. In addition, the heart homogenate was filtered through two layers of cheesecloth before centrifugation. The precipitates thus obtained were resuspended using a Polytron (setting 6; 3 sec) in 10 mM Tris (pH 7.5), 1 mM EDTA, 0.1% digitonin (lot 16F2081; Sigma Chemical Co., St. Louis, MO), 0.02% cholate at a protein concentration of approximately 3 mg/ml. In our hands, the corresponding amounts of tissue used (in a final volume of 5 ml) were as follows: one hemisphere of cerebral cortex; six pairs of striata; six pairs of hippocampi; one cerebellum; and one heart. The homogenates were centrifuged at 40,000 × *g* in a Sorvall centrifuge for 20 min. The precipitate was resuspended (Polytron setting of 6; 3 sec) in the original volume (e.g., 5 ml) of 10 mM Tris (pH 7.5), 1 mM EDTA, 1% digitonin, and 0.2% cholate and incubated on ice for 30 min. The homogenates were then centrifuged at 150,000 × *g* for 40 min. The supernatants thus obtained were used immediately as solubilized receptor preparations.

Generation of antisera. The peptides shown in Table 1 (except peptide 2) were synthesized by Dr. Ruth Angeletti (Department of Pathology, University of Pennsylvania, Philadelphia) using standard methodology (20). Peptide 1 was coupled to KLH or BSA using MBS (21). The protein and MBS were incubated for 1 hr at room temperature, then free MBS was removed after chromatography over Sephadex G-50. The protein-MBS (1 mg) and peptide (1 mg) were combined and incubated for 2 hr at 23°. Protein-bound and free peptide were separated over a column of Sephadex G-50. Peptide coupled to KLH (0.1 mg in 50% Freund's complete adjuvant) was injected into New Zealand white rabbits, using a multiple subcutaneous and intramuscular injection protocol (22). After 1 month the rabbits were boosted with 0.1 mg of the peptide-KLH conjugate in 1 ml of Freund's incomplete adjuvant, with a similar injection protocol. Antisera (anti-m_1 antisera) were collected each month after the booster injection and initially screened in a solid phase radioimmunoassay. Peptide 1 (2.5 μg) or peptide 1-BSA conjugate (5 μg) were dried (overnight at 37°) into wells of microtiter plates (Falcon MicroTest III). The wells were blocked using 1% BSA in TBS (20 mM Tris, pH 7.5, 500 mM NaCl), then washed three times with TBS. Dilutions of antisera (1:100–1:100,000) in TBS were added to each well and incubated for 2 hr at room temperature or overnight at 4°. Wells were rinsed three times with TBS, and ^{125}I-goat anti-rabbit secondary antibody (50,000 cpm/well) in 1% BSA/TBS was added. After 2-hr incubation at room temperature, wells were rinsed three times with TBS and bound ^{125}I was quantified.

Antisera that contained antipeptide antibody were precipitated by addition of an equal volume of 100% saturated $(NH_4)_2SO_4$ and centrifugation at 10,000 × *g*, dialyzed overnight against 10 mM sodium phosphate (pH 7), lyophilized, and stored frozen at −22° or −80°. This procedure was found to stabilize the antibody, as dramatic losses in the ability of the antisera to immunoprecipitate muscarinic receptors were seen after freezing of crude antisera at −22°, even though these antisera retained high titer in the solid phase radioimmunoassay. Aliquots of lyophilized antisera were dissolved as needed in 0.1 M potassium bicarbonate (pH 8) or TBS for subsequent use. Unless otherwise noted, the solution was made to 10 mg of lyophilized antiserum/ml, then diluted 10-fold into the precipitation assay.

A monoclonal antibody (31-1D1) to the cardiac muscarinic receptor was a generous gift of Dr. Neil M. Nathanson (University of Washington, Seattle) and has been described in detail elsewhere (23).

Immunoprecipitation. Immunoprecipitations of [^{3}H]QNB and [^{3}H]PZ binding activities were carried out in 0.1–0.5-ml volumes. Solubilized receptor (0.9 volumes) and 0.05 volume of [^{3}H]QNB (final concentration, 5 nM) or [^{3}H]PZ (final concentration 75 nM) were incubated for 20 min at 30°, the mixture was placed on ice, and antibody, buffer, or competing peptide (0.05 volume) was added to give final concentrations as indicated. Samples were incubated at 4° for 16–20 hr. When peptides were used to compete for antibody binding, antibody and peptide were preincubated (20 min at 30°) before addition to receptor. Sample volumes were brought to 0.5 ml using 10 mM Tris (pH 7.5), 1 mM EDTA, 0.1% digitonin, 0.02% cholate, and 0.9% NaCl, then were desalted over 3-ml columns of Sephadex G-50 in the same buffer to a final volume of 1 ml. Pansorbin (Calbiochem, LaJolla, CA) was stripped by boiling for 30 min in 3% SDS, 1% mercaptoethanol, followed by five washes with TBS. Stripped pansorbin (200 μl of a 10% slurry) or 100 μl of goat anti-mouse IgG_1-agarose (Sigma) were added, and samples were incubated for 45 min at 4° (m_1 antisera) or 4 hr at 4° (31-1D1 antibody). Precipitates were collected by centrifugation in a microcentrifuge. Pellets were washed with 0.5 ml of 10 mM Tris (pH 7.5), 1 mM EDTA, 0.1% digitonin, 0.02% cholate, and 0.9% NaCl, then were suspended in 50 μl of 3% NaOH/3% deoxycholate. Samples were transferred to counting vials after addition of 0.5 ml of H_2O.

Binding assay. [^{3}H]QNB and [^{3}H]PZ binding to solubilized receptors was performed using assay conditions essentially as described (24), with the exception that incubation of receptor and ligand was performed at 30° for 20 min followed by an overnight incubation at 4°. The final incubation volumes were identical to the volumes used during the respective immunoprecipitations. Bound and free ligand were separated by chromatography over Sephadex G-50 as described above, at the appropriate time relative to the immunoprecipitation. Atropine (1 μM) was used to define nonspecific binding.

Results

Table 1 shows the structures of the peptides used in this study. Structures 1–4 represent C-terminal regions of rat muscarinic acetylcholine receptors, using the nomenclature of Bonner *et al.* (15). Rabbit antisera were generated using peptide 1 conjugated to KLH. The structure of peptide 1 was modified to include a cysteine at the amino instead of the carboxyl terminus, to permit coupling with MBS but to reduce oxidation of

TABLE 1

Structures of peptides found in the C-terminal regions of rat muscarinic receptors

The structures of peptides 1–4 are based on sequence data obtained in rats from several groups (see text). The nomenclature used for m_1–m_4 muscarinic receptor subtypes is that of Bonner *et al.* (15). Amino acid sequences included in the peptide structures correspond to the following numbered amino acids: m_1, 443–459; m_2, 448–466; m_3, 561–578; and m_4, 460–478.

Peptide	Peptide structure*	Receptor subtype
1	CRKIPKRPGSVHRTPSRQ	m_1
2	KKTFHLLMCHYKNIGATR	m_2
3	QCDKRKRRKQQYQQRQSV	m_3
4	KKTFRHLLLCQYRNIGTAR	m_4

* Standard amino acid abbreviations are used.

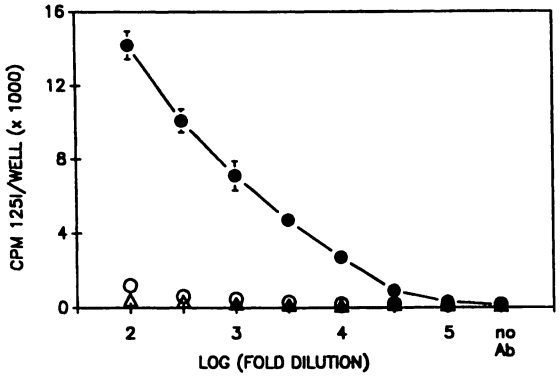

Fig. 1. Solid phase radioimmunoassay of rabbit anti-m_1 antisera. Rabbits were injected with peptide 1-KLH conjugate. After one booster injection, the serum was obtained and screened in a solid phase radioimmunoassay as described in Materials and Methods. ●, Anti-m_1 antiserum, screened against peptide 1; ○, anti-m_1 antiserum, screened against the peptide TSVPPAVSPNITVLAP; △, normal rabbit serum, screened against peptide 1. The *x* axis indicates the final dilution of antibody (*no Ab*, no added antibody); the *y* axis indicates the total cpm of ^{125}I/microtiter well. Data are the average of triplicates ± standard deviation. The same titer against peptide 1 was obtained in two rabbits. For the purposes of this study, the serum of only one rabbit was used.

the cysteine during synthesis of the peptide. The anti-m_1 antisera obtained after one booster injection had high titer against peptide 1 or BSA-peptide conjugate in a solid phase radioimmunoassay (Fig. 1). The activity of normal rabbit serum against peptide 1, or anti-m_1 antisera against the peptide TSVPPAVSPNITVLAP (*N*-terminal amino acids 3–18 of the rat m_1 muscarinic receptor), in the solid phase radioimmunoassay were similar to that seen in the absence of added antibody (Fig. 1). The anti-m_1 antisera were precipitated using $(NH_4)_2SO_4$, lyophilized, and stored as described above.

Aliquots of lyophilized anti-m_1 antisera were screened for their ability to immunoprecipitate muscarinic receptors solubilized from rat forebrain. Both [^{3}H]QNB and [^{3}H]PZ were used to label solubilized forebrain receptors. Solubilized tissue and radioligand were incubated for 20 min at 30°, then 16 hr at 4°. When anti-m_1 antisera were included during the 4° incubation, [^{3}H]QNB and [^{3}H]PZ binding were immunoprecipitated from forebrain (pooled cortex, hippocampus, and striatum) (Fig. 2). Antibody did not inhibit [^{3}H]QNB binding to solubilized receptors. Dilutions of anti-m_1 antisera as low as 0.03 mg/ml immunoprecipitated [^{3}H]PZ and [^{3}H]QNB binding activity (Fig. 2). A final concentration of 1 mg/ml of the anti-m_1 antisera appeared to be optimal in the immunoprecipitation assay, as addition of higher amounts of anti-m_1 antisera (e.g.,

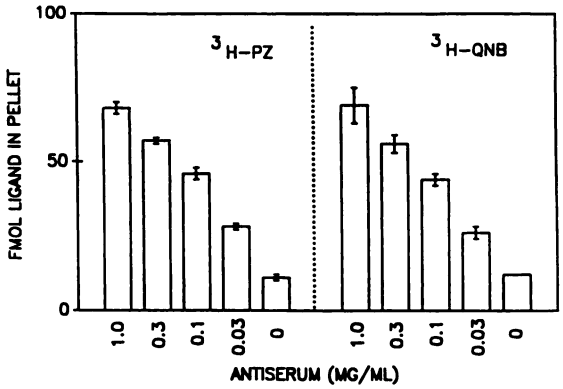

Fig. 2. Immunoprecipitation of [^{3}H]PZ and [^{3}H]QNB binding activity from rat forebrain. Solubilized forebrain (pooled cortex, striatum, and hippocampus) receptors were incubated with [^{3}H]QNB (8 nM) or [^{3}H]PZ (75 nM) for 20 min at 30°. The anti-m_1 antisera were added to give the final dilutions indicated (final volume, 0.25 ml) and incubations were continued for 16 hr at 4°. Samples were immunoprecipitated using pansorbin as described in Materials and Methods. Binding activities precipitated are presented as fmol of binding activity ± standard deviation of triplicates. These experiments have been repeated at least three times. At the time of precipitation with pansorbin there were 151 fmol/tube and 154 fmol/tube of [^{3}H]PZ and [^{3}H]QNB binding, respectively. Final antisera concentrations were as indicated (0 indicates no added antibody).

3.2 mg/ml) did not precipitate more [^{3}H]QNB binding activity than 1 mg/ml of the antisera, and generally a *reduced* amount of binding activity was precipitated using the higher amounts of antisera. In four experiments, 62 ± 26% (mean ± SE) of the binding activity precipitated using 1 mg/ml antisera was precipitated using 3.2 mg/ml antisera. Radioactivity precipitated in the absence of antisera and in the presence of 1:10 dilutions of fresh (or 1 mg/ml of lyophilized) preimmune or normal rabbit serum were identical. Addition of higher amounts of pansorbin, the use of protein A-agarose, or longer centrifugation times did not increase the amount of [^{3}H]QNB binding activity that could be immunoprecipitated (data not shown). Similarly, no significant amounts of binding activity could be immunoprecipitated after precipitation using anti-m_1 antisera (1 mg/ml final concentration) and reincubation of supernatants with additional anti-m_1 antisera (1 mg/ml final concentration), pansorbin, or their combination. [^{3}H]QNB recovered in the supernatants was confirmed to be receptor-bound by desalting. Neither [^{3}H]QNB nor [^{3}H]PZ binding activity could be immunoprecipitated from solubilized preparations of heart using the anti-m_1 antisera (Fig. 3, A and B), although significant binding activity could be precipitated from forebrain under identical conditions (Fig. 3, A and B). To determine whether a specific anatomical region of the forebrain preparation contained most of the precipitable binding sites, the forebrain was dissected into three component parts, cortex, hippocampus, and striatum. The ability of the anti-m_1 antisera to precipitate receptors was tested in each preparation. Fig. 3C demonstrates that similar amounts of [^{3}H]PZ binding activity could be precipitated from the individual dissections of cortex, striatum, and hippocampus.

To test the possibility that heart tissue somehow compromised the ability of the anti-m_1 antisera to immunoprecipitate radioligand binding activity, the following experiment was performed. Varying amounts of receptor solubilized from forebrain were incubated with or without a constant amount of receptor solubilized from heart, in the presence of anti-m_1 antisera. The amount of binding activity precipitated from solution was the

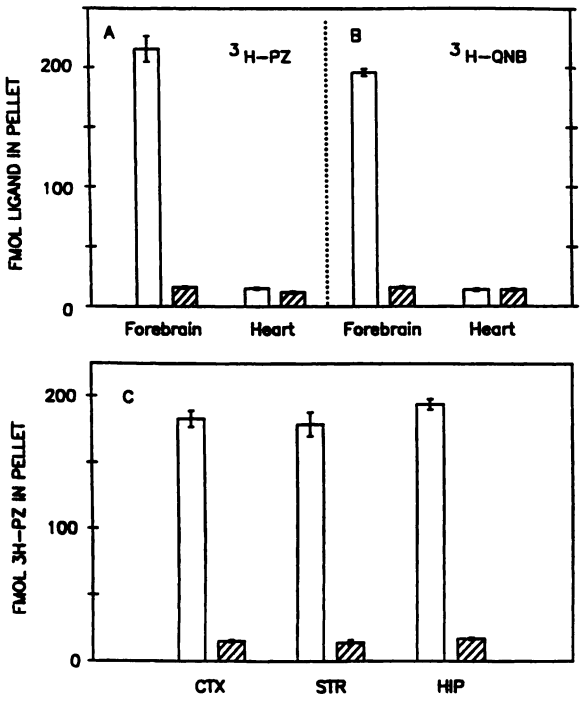

Fig. 3. Immunoprecipitation of [^{3}H]PZ and [^{3}H]QNB binding activities from brain and heart tissues. A, Receptors solubilized from forebrain or heart (425 μl) were incubated with 50 μl of [^{3}H]PZ (75 nM) for 20 min at 25°. Samples were placed on ice and 25 μl of anti-m_1 antisera (20 mg of protein/ml) or vehicle were added. After overnight incubation at 4°, samples were desalted and processed as described in Materials and Methods. Results are expressed as fmol precipitated per tube (average of triplicate determinations ± standard deviation). The amount of solubilized binding activity (fmol of [^{3}H]PZ bound) added per tube in each tissue was: forebrain, 860; heart, 390. Similar results were obtained in at least three separate experiments. ■, No antibody; □, plus antibody. B, A protocol identical to that described in A was followed using 8 nM [^{3}H]QNB instead of [^{3}H]PZ. The amount of solubilized binding activity (fmol of [^{3}H]QNB bound) added per tube in each tissue was: forebrain, 820; heart, 550. C, Methods were identical to A, except that [^{3}H]PZ binding activity was used in the immunoprecipitation assay. *CTX*, cortex; *STR*, striatum, *HIP*, hippocampus. ■, no antibody; □, plus antibody. The amounts of [^{3}H]PZ binding activity (fmol) added to the incubations were: cortex, 660; hippocampus, 670; striatum, 980.

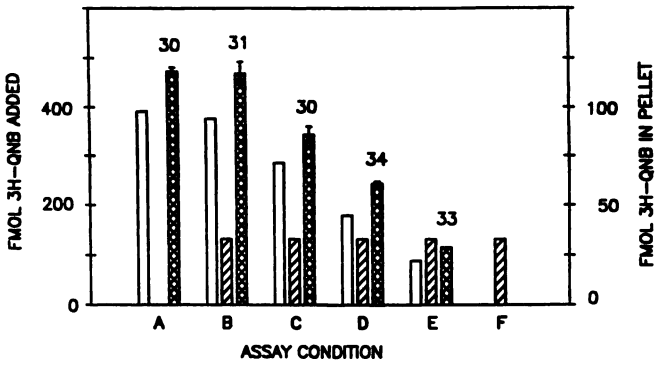

Fig. 4. Immunoprecipitation of mixtures of forebrain and heart receptor by anti-m_1 antisera. Buffer (condition A) or a single concentration of heart (condition B–F) were added to varying dilutions of solubilized forebrain, such that the final volumes of receptor were identical. Receptors were incubated with [^{3}H]QNB and anti-m_1 antisera (final concentration, 1 mg/ml) using the standard assay conditions. The *left ordinate* represents the amount of added receptor (average of duplicate determinations). The *right ordinate* represents the amount of receptor in the immunoprecipitate after subtracting no-antibody blank values (average of triplicates ± standard deviation). □, Added forebrain; ■, added heart; ■, precipitated. These experiments have been repeated twice. Condition A represents forebrain alone; B–E are forebrain plus heart; F is heart alone. The *numbers* over the *bars* represent the amount of activity precipitated as a percentage of added forebrain.

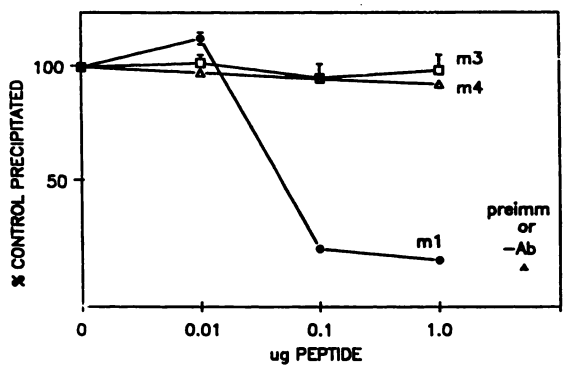

Fig. 5. Specificity of peptides to inhibit immunoprecipitation of [^{3}H]QNB binding activity from rat forebrain. Solubilized forebrain receptors were incubated with [^{3}H]QNB for 20 min at 25°, then overnight with anti-m_1 antisera (final concentration, 1 mg/ml) at 4°. Incubations included competing peptides as indicated. Samples were processed for immunoprecipitation as discussed in Materials and Methods. ●, Peptide 1; □, peptide 3; Δ, peptide 4; ▲, preimmune serum. Data are expressed as percentage of activity immunoprecipitated in the absence of added peptide and are the mean ± standard error of triplicate determinations. This experiment was repeated with similar results.

same in the absence or presence of heart tissue (Fig. 4, A and B), and correlated with the amount of added forebrain receptors (Fig. 4, B–F).

To verify further the specificity of the anti-m_1 antisera, solubilized receptors from forebrain were labeled with [^{3}H]QNB and incubated with antibody in the presence of various competing peptides. These peptides were derived from similar (C-terminal) regions of structurally related receptor proteins (see Table 1). Fig. 5 shows that the ability of the anti-m_1 antisera to immunoprecipitate [^{3}H]QNB binding activity from forebrain was blocked potently by peptide 1 but not by peptides 3 or 4. Note that the structure of peptide 2 is very similar to that of peptide 4; consequently, this peptide was not used in this experiment.

We attempted Western blot analysis of crude and of highly purified preparations of receptor from forebrain and heart, using the anti-m_1 antisera as a probe. Unfortunately, these antisera recognize some antigenic substance that produces a nonspecific 'ladder' of labeled bands, ranging from the dye front to approximately 120 kDa. This ladder is seen in lanes containing either protein or sample buffer alone with or without the use of reducing agents, does not reflect bands seen using silver staining, and is reproduced by antisera from two rabbits but not by their preimmune sera. We tried a variety of blocking agents and their combination, as well as several different types of secondary antibody (iodinated, biotinylated, and horseradish peroxidase-conjugated) to reduce this banding pattern, to no avail. Both crude and protein A-Sepharose-purified antisera generated this ladder at all dilutions tested (1:50–1:100). The antibodies producing this artifact were recovered in the non-absorbed fraction of a peptide 1-Affigel 10 column, whereas antibodies with high titer to peptide 1 in solid phase radioimmunoassay and having the ability to immunoprecipitate receptors were adsorbed by the peptide 1 resin. Antibodies with titer to the peptide could be eluted from the resin using glycine (pH 2.5) or 3 M $MgCl_2$, but these antibodies were virtually

inactive in the immunoprecipitation assay. Together, these factors currently prevent the use of the anti-m_1 antibodies in the Western blot protocol.

The monoclonal antibody 31-1D1, which was raised to the porcine cardiac muscarinic receptor (23), immunoprecipitated receptors solubilized from both heart and forebrain (Fig. 6). In four experiments using the 31-1D1 antibody, 67 ± 12% (mean ± SD) of added [^{3}H]QNB binding activity could be immunoprecipitated from heart using this antibody, whereas only 15 ± 6% of [^{3}H]QNB binding activity could be precipitated from forebrain (Fig. 6A). Although approximately 40% of added [^{3}H]PZ binding activity could be precipitated from heart, no detectable [^{3}H]PZ binding activity could be precipitated from forebrain using the 31-1D1 antibody (Fig. 6B).

Discussion

Many physiological and radioligand binding studies have supported the concept that subpopulations of muscarinic receptors can be discriminated in mammalian tissues (1, 4). In general, these studies have relied on the ability of PZ, AF-DX116, and a few other drugs to interact selectively with muscarinic receptors in various tissues (1–5, 25, 26). Purification of muscarinic receptors from pig heart (12) and pig and rat forebrain (13, 14) has allowed the application of molecular biological techniques to identify the primary amino acid sequences of muscarinic receptors present in those tissues. The results of molecular cloning studies using cDNA and genomic libraries derived from rats, humans, and pigs are consistent with the concept that at least four subtypes of muscarinic receptors exist. This conclusion is based on the identification of four homologous cDNA sequences coding for proteins that bind muscarinic ligands with high affinity. Studies using cDNA clones expressed in Cos7 cells (15, 16) *Xenopus* oocytes (17, 27), and A9L cells (28) indicated that two of the clones [termed m_1 and m_4 by Bonner *et al.* (15)] coded for proteins that bound PZ with high affinity, based on unlabeled PZ inhibiting [^{3}H]QNB or [^{3}H]*N*-methyl scopolamine binding. The other two clones coded for receptors that had somewhat (m_3) or markedly (m_2) lower affinity for PZ in the binding assay. Electrophysiological data (27) suggested that the m_1 and m_2 subtypes, when expressed in *Xenopus* occytes, provoked unique electrical responses. On the other hand, the cardiac m_2 receptor, when expressed in CHO cells, modulated both the adenylate cyclase activity and inositol polyphosphate breakdown, albeit with greatly different efficiencies (29). Characterization of the distribution of mRNA encoding m_1–m_4 subtypes of muscarinic receptor using Northern blot analysis (16–18) demonstrated the expression of mRNA for all of the subtypes in cortex and mRNA for the m_2 subtype in heart, as well as selective tissue distributions of the other subtypes. Similarly, experiments utilizing *in situ* hybridization to mRNA in brain pointed to selective (but not exclusive) expressions of the mRNA for m_1, m_3, and m_4 receptor subtypes in different regions of rat brain (15, 30).

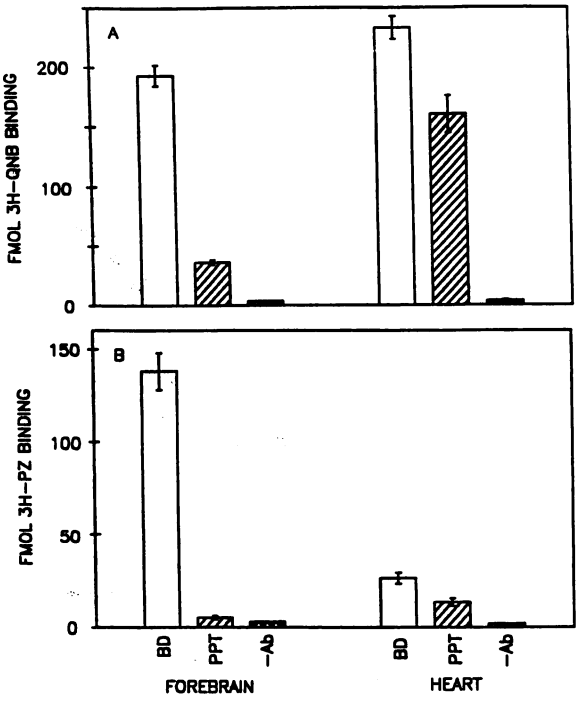

Fig. 6. Immunoprecipitation of muscarinic binding activity from brain and heart using a cardiac-selective antibody. A, The monoclonal antibody 31-1D1 (final concentration, 1:1000 dilution of ascites fluid) was incubated with receptors solubilized from forebrain (*left*) or heart (*right*). Data are expressed as fmol of [^{3}H]QNB and are the average of duplicates (*error bars* indicate range). These experiments have been repeated three to four times. □, Added [^{3}H]QNB bound to receptor; ■, [^{3}H]QNB precipitated in the presence of antibody; ■, amount of [^{3}H]QNB precipitated in the absence of antibody. Values of percentage of binding activity precipitated are given in the text. No further activity could be precipitated from heart using higher dilutions of ascites fluid or additional goat anti-mouse-agarose. B, Conditions were the same as in A except that [^{3}H]PZ was used as the radioligand.

Based on cDNA sequence data, the m_1–m_4 muscarinic receptors have been shown to be highly homologous in primary amino acid sequence, but the homology is greatest in seven putative membrane-spanning regions (see Ref. 31 for discussion). In other regions, such as the third cytoplasmic loop and the N- and C-termini, the sequences have diverged. Previous studies using antibodies have suggested a conservation of antigenic epitopes between the muscarinic receptors from a variety of sources and were unable to reveal antigenic differences (32–34).

Recently, however, Luetje *et al.* (23) demonstrated that monoclonal antibodies to purified preparations of cardiac muscarinic receptors could immunoprecipitate [^{3}H]QNB binding activity from heart but not from several brain regions. PZ shows a uniform and low affinity (K_d = 1 μM) for muscarinic receptors in membrane preparations from rat or pig heart (1) which have thus been termed M_2 muscarinic receptors. It is of interest that, upon solubilization, cardiac muscarinic receptors express a high affinity (K_d = 10 nM) component of PZ binding not seen in cardiac membranes (35–37). The molecular basis for this observation remains uncharacterized. One goal of the present study was to determine whether [^{3}H]PZ labeled more than a single receptor subtype in brain and heart.

To address this question, antisera were generated to a synthetic oligopeptide with a structure nearly identical to the C-terminal amino acid sequence of the m_1 muscarinic receptor subtype. Comparing this peptide sequence with C-terminal sequences published for rat, pig, and human m_1–m_4 muscarinic receptors (15–19), the hamster and human β_2-adrenergic receptors (38–40), the avian and human β_1-adrenergic receptors (41, 42), and the human platelet α_2-adrenergic receptor (43) shows it to be unique to the m_1 muscarinic receptor. In addition, this

sequence is conserved between pig, rat, and human m_1 muscarinic receptors. The rabbit anti-m_1 antisera immunoprecipitated [^{3}H]QNB binding activity from brain, and this was inhibited by peptide 1 but not by peptides 3 or 4 (Fig. 5). [^{3}H]PZ has been shown to label a population of muscarinic binding sites in solubilized preparations of brain (24, 44) and heart (35–37). The anti-m_1 antisera were capable of immunoprecipitating [^{3}H]PZ binding activity solubilized from forebrain regions, but not heart (Figs. 2 and 3). Taken together, these observations indicate that [^{3}H]PZ binds with high affinity to more than one subtype of muscarinic receptor, at least in solubilized preparations. These data also suggest that forebrain contains a population of m_1 muscarinic receptors, in addition to other receptors that can be labeled using [^{3}H]PZ but not precipitated using anti-m_1 antisera. Similarly, heart contains a population of muscarinic receptors that can be labeled using [^{3}H]PZ and precipitated using the 31-1D1 antibody but not precipitated using anti-m_1 antisera.

In addition to the more likely explanation that [^{3}H]PZ labels more than the m_1 subtype of muscarinic receptor, there are several other possible explanations for these results. For example, antigenic determinants in solubilized preparations of different tissues might be uniquely masked by detergent, aggregation, or intrinsic conformational states of the receptor. It is also possible that, during the overnight incubation of antisera with receptor, proteolysis of antigenic determinants on the receptor occurred but the proteolysis did not affect ligand binding. However, we found no effect of a cocktail of protease inhibitors on the ability of anti-m_1 antisera to immunoprecipitate forebrain and heart receptors and also no effect of these inhibitors on the banding pattern after SDS-polyacrylamide gel electrophoresis of [^{3}H]propylbenzilylcholine mustard-labeled receptors.[1] Any proteolysis that did occur must have been highly selective for heart receptors inasmuch as during coincubation of heart and forebrain receptors the m_1 antisera retained the ability to immunoprecipitate binding activity (Fig. 4). Similarly, a reverse selectivity of the ability to immunoprecipitate from heart and forebrain was seen using different antibodies (Fig. 2 versus Fig. 6). On the other hand, it has been shown using site-directed mutagenesis (45) and proteolytic treatments (46, 47) of β-adrenergic receptors that significant deletions of primary amino acid sequence can occur with little change in antagonist binding activity.

Another possibility that appears consistent with our data is that both [^{3}H]QNB and [^{3}H]PZ bind to more than one subtype of muscarinic receptor in rat tissues. Although this is rather well established for [^{3}H]QNB, to date the lack of knowledge of the actual distribution of the m_1–m_4 muscarinic receptor subtypes in rat tissues has precluded a direct examination of this issue for [^{3}H]PZ. Based on the ability of cell lines containing cDNA clones for the m_1–m_4 receptors to express high and low affinity PZ binding activities (15, 16, 28), it is clear that two subtypes (m_1 and m_4) and possibly a third (m_3) may bind PZ with high affinity. Additionally, it now seems likely that more than four subtypes of muscarinic receptor may exist, which may have high or low affinity for PZ (15). Again, whether any or all of these subtypes are expressed in tissues such as rat brain remains to be determined. Peralta *et al.* (16) have recently used Northern blot analysis to demonstrate that the mRNAs for the m_1–m_4 muscarinic receptor subtypes are expressed in brain and other rat tissues. Using radioligand binding techniques, we have found[2] that, under the assay conditions used in this paper (e.g., 16-hr incubation at 4°), Scatchard plots of [^{3}H]PZ binding in forebrain tissues appear curvilinear. These data make an accurate determination of the absolute levels of PZ binding sites, relative to QNB binding sites, difficult in this tissue. However, [^{3}H]PZ binding levels approach 80% of QNB binding levels even under these conditions, and thus the observation that similar fractions of PZ and QNB binding activity were precipitated from forebrain using anti-m_1 antisera is not totally unexpected. Without an absolute knowledge of the relative levels of m_1–m_4 subtype in cortex, and with the variability inherent in the assay, it is impossible to predict the fractions of [^{3}H]PZ and [^{3}H]QNB binding activity expected to be precipitated by the anti-m_1 antisera in forebrain regions. Currently, then, we cannot discuss the antibody data presented in this manuscript in terms of the *quantitative* distribution of muscarinic receptor subtypes in brain. In preliminary experiments we have found that the anti-m_1 antisera immunoprecipitate [^{3}H]QNB-labeled receptors from CHO cells transfected with a cDNA clone for the m_1 receptor subtype (16) (received as a gift from Dr. D. Capon, Genentech, South San Francisco, CA). Receptors could not be precipitated from CHO cells transfected with cDNA for the m_2 receptor subtype, nor from SKNSH, 132-1N1, or NG108 cells. Based on Northern blot analysis, the latter three cell lines contain the m_3, m_3, and m_4 subtypes of receptor, respectively. In the absence of Western blot capabilities, these data, together with data obtained in heart and forebrain, support the concept that our antibodies at least can distinguish between subtypes of the muscarinic receptor, although they possibly recognize more than one protein present in cellular homogenates. We believe that the use of similar antipeptide antisera to other subtypes of the muscarinic receptor, combined with radioligand binding techniques, may be useful in future studies designed to examine quantitatively the distribution of muscarinic receptor subtypes in brain.

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[2] G. Luthin, unpublished observations.

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