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Scanning Mutagenesis Identifies Amino Acid Side Chains in Transmembrane Domain 5 of the M_1 Muscarinic Receptor that Participate in Binding the Acetyl Methyl Group of Acetylcholine

KAREN ALLMAN,[1] KAREN M. PAGE,[2] CAROL A.M. CURTIS, and EDWARD C. HULME

Division of Physical Biochemistry, Medical Research Council National Institute for Medical Research, London, United Kingdom

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ABSTRACT

The exofacial part of transmembrane domain 5 (TMD 5) of the cationic amine-binding subclass of 7-transmembrane receptors is thought to be important in binding the side chain of the agonist. Residues Ile-188 through Ala-196 in TMD 5 of the M_1 muscarinic acetylcholine receptor (mAChR) have been studied by Cys- and Ala-scanning mutagenesis. The results are consistent with a helical conformation for this sequence. The positively charged sulfhydryl reagent *N*-trimethyl-2-aminoethyl methanethiosulfonate reacted selectively with Phe-190 → Cys, Thr-192 → Cys, and Ala-193 → Cys, indicating that the face of TMD 5 accessible from the binding site crevice is consistent with a recent model by Baldwin and colleagues of the transmembrane domain of the 7-transmembrane receptors. In contrast, the acetylcholine derivative bromoacetylcholine reacted selectively with Thr-192 → Cys, which forms the focus of a group of amino acids (Ile-188, Thr-189, Thr-192, Ala-196) whose mutation decreased the binding affinity of the transmitter ACh itself. The center of this patch of residues is offset to one side of the binding pocket, suggesting that a rotation of TMD 5, relative to that implied by the Baldwin model, may be necessary to optimize the anchoring of acetylcholine within the binding site of the M_1 mAChR. An induced rotation of TMD 5 could contribute to the formation of the activated state of the receptor.

Amino acids located in the exofacial region of the transmembrane domain (TMD) of the cationic amine-binding subclass of G protein-coupled receptors (GPCRs) are important for agonist binding (for review, see Strange, 1996). Most of the experimental data has come from mutations that reduce the binding affinities of specific ligands. Such information can suggest, but not prove, the existence of a direct receptor-ligand contact, because indirect conformational effects of mutations on the ligand binding site cannot be excluded (Shortle, 1992; Colquhoun, 1998).

In TMD 5 of the cationic amine receptors, the sequences that lie near or within the outer leaflet of the phospholipid bilayer typically contain several polar amino acids that vary according to the pharmacology of the receptor. Catecholamine receptors contain two or three serines at positions 5.42, 5.43, and 5.46, using the standard nomenclature of Ballesteros and Weinstein (1995). These participate in the ligation of the catechol *m*- and *p*-hydroxyl groups, although the pattern varies according to the receptor type (Strader et al., 1989; Wang et al., 1991; Mansour et al., 1992; Pollock et al., 1992; Cavalli et al., 1996; Hwa and Perez, 1996; Wetzel et al., 1996; Wiens et al., 1998; Sartania and Strange, 1999). In the 5-HT1A receptor positions Ser-5.42 and Thr-5.43 may interact with the 5′-hydroxyl group, whereas in the 5-HT6 receptor Thr-5.46 may interact with the indole nitrogen of 5-HT (Ho et al., 1992; Boess et al., 1997). In the histamine H_1 receptors, Asn-5.46 is important for the binding of histamine (Ohta et al., 1999), whereas position 5.42 is relatively unimportant. In the H_2 receptors, Asp-5.42 and Thr-5.46 have both been suggested to interact with the imidazole ring of histamine (Gantz et al., 1992).

The muscarinic acetylcholine receptors (mAChRs) contain one threonine at position 5.42, and a second at 5.39, whereas positions 5.43 and 5.46 are occupied by alanines. Positions 5.39 and 5.42 have been investigated in the M_3 mAChR by mutation to alanine (Wess et al., 1991), in a study in which the mutants were probed with a series of acetylcholine (ACh) analogs, in which various elements of the ACh ester linkage were substituted by methylene groups or by a sulfur atom

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[1] Current address: SmithKline Beecham Pharmaceuticals, New Frontiers Science Park (South), Third Ave., Essex CM19 5AW, UK.

[2] Current address: Department of Pharmacology, University College London, London WC1E 6BT, UK.

ABBREVIATIONS: TMD, transmembrane domain; ACh, acetylcholine; AChR, acetylcholine receptor; MAChR, muscarinic acetylcholine receptor; NMS, (−)-*N*-methylscopolamine; BrACh, bromoacetylcholine; MTSET, *N*-trimethyl-2-aminoethylmethanethiosulfonate; MTSES, 2-sulfonylethylmethanethiosulfonate; MTSEA, 2-aminoethylmethanethiosulfonate; PI, phosphoinositide; SH, sulfhydryl.

(Wess et al., 1992). Position 5.42 has also been examined by alanine substitution in the M_1 mAChR (Huang et al., 1999). In general, the results supported the conjecture that Thr-5.39 and Thr-5.42 are important in defining the binding site for the ACh side chain. However, both "productive" and "nonproductive" modes of binding of ACh analogs exist, and replacement of polar by nonpolar moieties within the side chain of ACh can alter the balance between these modes of binding (Page et al., 1995). For this reason, it has not been possible to identify specific interactions between the Thr residues and moieties within the ACh molecule using alanine mutants alone.

In this study, we report an alternative approach. It is based on cysteine substitution of all of the amino acids between Ile-188 and Ala-196 (positions 5.38–5.46) of the rat M_1 mAChR, followed by chemical reaction of the mutant receptors with a variety of sulfhydryl-directed reagents with reactive side chains. A similar approach has been applied to TMD 5 of the α_{2A}-adrenergic receptor (Marjamäki et al., 1998, 1999).

In addition to positively and negatively charged methanethiosulfonate derivatives (Javitch et al., 1995), we have used a derivative of ACh, bromoacetylcholine (BrACh), in which halogenation of the acetyl methyl group creates a compound with the potential to alkylate sulfhydryl groups.

The ability of BrACh to block the binding site of the M_1 mAChR was strongly enhanced after the introduction of sulfhydryl groups into TMD 5 by Cys substitution of particular amino acids. Selective blockade of the Thr-192 → Cys mutant by BrACh suggests that the alkylating side chain of the affinity label can contact position 5.42. Thus, the threonine methyl group at position 192 may be positioned to make a Van der Waals contact with the acetyl methyl group of ACh in the wild-type receptor. Substitution mutagenesis experiments support the view that this residue and its neighbors contribute to a binding pocket for the acetyl methyl group of ACh. This may account for the high affinity and potency of ACh relative to formylcholine. The comparison of functional with binding data suggests that the interactions made by residues in TMD 5 are more important for the binding of ACh than for the induction of the conformational change that leads to phosphoinositide (PI) signaling. Preliminary accounts of some of these experiments have been published (Allman et al., 1997).

Experimental Procedures

Methods. The experimental procedures were performed as described previously. Briefly, residues 188 through 196 of the rat M_1 mAChR were individually mutated to Cys, Ala, Gly, or Ser (Ala itself was changed to Gly, and Gly to Ala) using a polymerase chain reaction method (Lu et al., 1997) or the Amersham phosphorothioate method (Page et al., 1995). Mutant receptors, cloned into the pCD expression vector, and verified by dideoxy sequencing, were transiently expressed in COS-7 cells following transfection by electroporation. Membrane preparations were made 3 days after transfection (Jones et al., 1995)

Binding of (−)-*N*-[^{3}H]methyl scopolamine ([^{3}H]NMS), ACh, and other ligands was measured at 30°C in a buffer containing 20 mM Na-HEPES, 100 mM NaCl, and 1 mM $MgCl_2$, pH 7.5, using an incubation time of 60 min (Jones et al., 1995). Alternatively, measurements were carried out at pH 8.0 in a buffer containing 20 mM EPPS. Nonspecific binding was defined with 1 μM atropine. Competition experiments were performed with 0.3 nM [^{3}H]NMS. In experiments in which guanine nucleotide shifts were studied, 1 mM $MgCl_2$ was replaced by 10 mM $MgCl_2$. PI turnover, stimulated by ACh, was assayed in Krebs-bicarbonate solution following prelabeling of the cells with [^{3}H]inositol (Jones et al., 1995).

Data analysis was performed using the program Sigma Plot 3.03 (SPSS Inc.). Binding curves were fitted to the Hill equation, or to one-site, two-site, or ternary complex models of binding, and affinity constants were corrected for the Cheng-Prusoff shift, as necessary (Page et al., 1995). PI dose-response curves were fitted to a four-parameter logistic function, yielding EC_{50} and E_{max} values. Values of signaling efficacy, adjusted for variations in receptor expression, were calculated as described (Hulme and Lu, 1998; Lu and Hulme, 1999; Ward et al., 1999).

Expression of receptor protein was assessed by immunocytochemical visualization of transfected cells using an immunoaffinity-purified antiserum raised against the C-terminal 13 amino acids of the sequence, as described (Lu et al., 1997).

Materials. [^{3}H]NMS (85 Ci/mmol), *myo*-D-[^{3}H]inositol (80 Ci/mmol), and the phosphorothioate mutagenesis kit were from Amersham Pharmacia Biotech. BrACh was purchased from Research Biochemicals Incorporated (Natick, MA). Methanethiosulfonate reagents were from Toronto Research Chemicals. Formylcholine was synthesized as described previously (Page et al., 1995). Other materials were of the highest commercial grade available.

Results

Binding of [^{3}H]NMS

Of the mutations of Ile-188 through Ala-196, in TMD 5 of the M_1 mAChR, only those of Ala-193 significantly affected the affinity of the receptor for the antagonist [^{3}H]NMS, causing reductions of 3.6-fold (Ala-193 → Cys) and 2.6-fold (Ala-193 → Gly) with respect to the wild-type (pK_d = 9.9; Table 1). Assays with a second antagonist, (−)-[^{3}H]quinuclidinyl benzilate did not show a reduction in affinity (pK_d = 10.6 for wild-type; 10.6 for Ala-193 → Gly; 11.0 for Ala-193 → Cys). Expression of the mutant receptors was reduced to less than 30% of the wild-type level only in the case of Met-194 → Cys (12–27%). Thus, with this possible exception, none of the

TABLE 1
Binding of [^{3}H]NMS to wild-type and TMD 5 mutant M_1 mAChRs
Saturation binding assays were performed on membrane preparations from COS-7 cells transfected with the wild-type and TMD 5 mutant M_1 mAChRs. Data were analyzed using a one-site model of binding. Results are mean ± S.E. of *n* experiments. The expression level of the wild-type receptor was 1.10 ± 0.12 pmol/mg of protein.

Mutant	pK_d	*n*	Expression
	−log M		*% WT*
WT	9.91 ± 0.02	8	100
I188C	10.00 ± 0.05	4	26 –41
I188A	10.11 ± 0.04	3	39 –55
T189C	9.96 ± 0.05	4	34 –52
T189S	9.80 ± 0.02	3	50 –85
F190C	9.92 ± 0.08	3	33 –40
F190A	9.98 ± 0.06	3	82 –87
G191C	9.87 ± 0.07	3	56 –64
G191A	9.98 ± 0.11	3	27 –51
T192C	9.96 ± 0.07	5	50 –64
T192A	9.68 ± 0.06	4	99–120
A193C	9.29 ± 0.04*	4	45 –49
A193G	9.47 ± 0.03*	4	20 –56
M194C	9.88 ± 0.03	3	12 –27
A195C	9.89 ± 0.05	3	51 –59
A195G	9.83 ± 0.10	4	63 –72
A196C	9.76 ± 0.08	4	80 –85
A196G	10.24 ± 0.06	4	83–110

* $P < .001$ with respect to wild-type M_1 mAChR.

mutations strongly affected the expression of the receptor or its processing to a form capable of high-affinity antagonist binding. These results were confirmed by immunocytochemical visualization of the expressed protein using an anti-C-terminal antibody (Lu et al., 1997); (data not shown).

Binding of ACh

The binding of ACh to the wild-type M_1 receptor expressed in COS-7 cells was characterized by a slightly flattened binding isotherm (n_H = 0.88), and a pIC_{50} of 4.9. The binding of ACh to the wild-type receptor showed little if any GTP sensitivity in these experiments (data not shown). The effects of the mutations of Ile-188 through Ala-196 are summarized in Fig. 1a. Mutations of four residues, Ile-188, Thr-189, Thr-192, and Ala-196, caused significant reductions in ACh affinity, the largest effects being slightly over 10-fold (Thr-192 → Cys, Ala-196 → Cys). Where comparisons could be made, the effects of other mutations at the same positions (Ile-188 → Ala, Thr-192 → Ala, and Ala-196 → Gly) were similar, but slightly smaller.

In contrast, the mutation Phe-190 → Cys caused a 6-fold increase in ACh affinity, and a reduction in the Hill coefficient (n_H = 0.70) (Fig. 2a). This effect was not seen for the corresponding Ala substitution.

Binding of Other Agonists

The effects of the Cys mutations of Ile-188, Thr-189, Phe-190, Thr-192, and Ala-196 on the binding affinities of two other muscarinic agonists of high efficacy, carbachol and oxotremorine-M were qualitatively similar to but quantitatively smaller than their effects on the binding of ACh (carbachol: 3-fold increase at Phe-190 → Cys, 7-fold reduction at Thr-192 → Cys, 3-fold reduction at Ala-196 → Cys; oxotremorine-M: 2-fold increase at Phe-190 → Cys, 4-fold reduction at Thr-192 → Cys, 8-fold reduction at Ala-196 → Cys). The affinity of formylcholine, which lacks the terminal methyl group of ACh, showed less than a 3-fold change in response to Cys mutations of Ile-188, Thr-192, and Ala-196, and less than a 2-fold reduction on making the corresponding Ala or Gly substitutions (Table 2). The affinity of the oxadiazole agonist L-698,583 (Ward et al., 1999) was reduced only 3-fold by the Thr-192 → Cys mutation, and this compound was not investigated further.

Effects on the PI Response

ACh elicits a robust PI response from COS-7 cells transfected with wild-type M_1 mAChRs, with an EC_{50} value of 0.1 μM, and an E_{max} value equivalent to three times the basal activity. The effects of the TMD 5 mutations on the ACh-induced PI response mediated by the M_1 mAChR were parallel to their effects on the binding of the agonist (Fig. 1b). Significant reductions in ACh potency resulted from mutations of Thr-192 (20- to 40-fold). Decreases also followed from the mutations of Ile-188, Thr-189, and Ala-196. As in the case of ACh binding, the effects of the Cys mutations were generally similar to those of the other mutations made at these sequence positions; replacement of Ala-196 by Gly had a somewhat larger effect than replacement by Cys. The maximum responses elicited by the mutant receptors were similar to wild-type (Fig. 1b). Calculations of signaling efficacy were performed as described previously (Hulme and Lu, 1998; Lu and Hulme, 1999; Ward et al., 1999). These assess the effects of mutations on the functional potency relative to the binding affinity, taking into account changes in receptor expression level. They showed a 3-fold decrease relative to wild-type for Thr-192 → Ala, and a 6-fold decrease for Ala-196 → Gly (Fig. 1b).

The mutation Phe-190 → Cys resulted in a 10-fold increase in ACh potency, again in parallel to the binding assay (Fig. 2b). Calculation of the signaling efficacy suggested a 4-fold increase relative to the wild-type receptor. This mutation also significantly increased the agonist-independent activity of the receptor, to 17% of the total signal. The enhanced basal

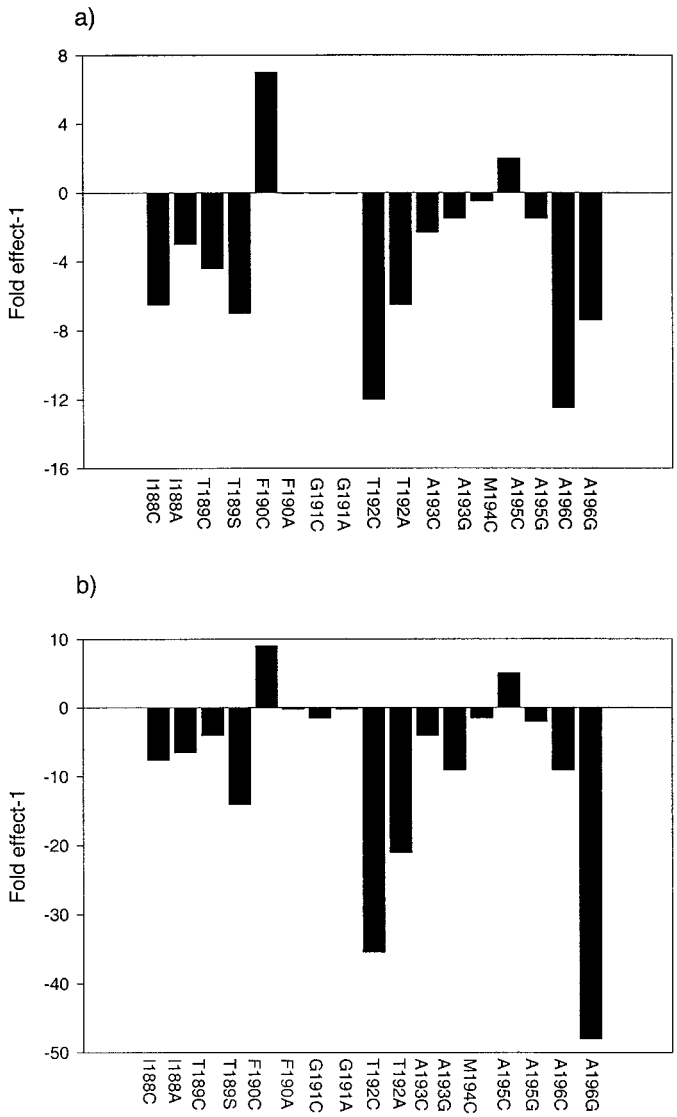

Fig. 1. Effects of mutations in TMD 5 of the M_1 mAChR on the binding affinity and signaling potency of Ach. Results are the mean ± S.E. of three or more determinations. Bars represent fold effect-1, relative to the wild-type control. Both decreases and increases are shown. a, effects on the affinity of ACh. The affinity of ACh for the wild-type receptor was 8×10^4 M^{-1}. The Hill coefficient of the wild-type binding curve was 0.88 ± 0.04 and was unaltered except for Thr-189 → Cys (0.66 ± 0.03) and Phe-190 → Cys (0.70 ± 0.03). b, effects on the potency of ACh in the PI assay. The pEC_{50} value of ACh at the wild-type receptor was 6.98 ± 0.06. The slope factors of the PI dose-response curves were generally close to 1.0. The E_{max} values ranged from 82 ± 2% (Thr-192 → Cys) to 130 ± 13% (Ala-193 → Gly). Calculations of signaling efficacy, as described previously (Lu and Hulme, 1999; Ward et al., 1999), showed decreases of 4-fold for Thr-192 → Ala, 7-fold for Ala-196 → Gly, and an increase of 4-fold for Phe-190 → Cys. All other values were within a factor of 3 of the control.

activity could be consistently inhibited by preincubation of the cells for 24 h with the muscarinic antagonists atropine and NMS (Fig. 2c), as reported for other constitutively active mutants (Lu and Hulme, 1999). The maximum PI response elicited by the partial agonist pilocarpine was also significantly increased from 72% of the maximal ACh response in the case of the wild-type receptor to 96% of the response for the Phe-190 → Cys mutant (Fig. 2d). The Phe-190 → Ala mutant did not exhibit raised basal activity.

Blockade by Sulfhydryl Reagents

BrACh. BrACh is a sulfhydryl-alkylating derivative of ACh. The ionization constants of most protein thiols are between 8 and 10. BrACh reacts with the thiolate anion, so reactions with BrACh were carried out at pH 8.0 in an attempt to ensure the ionization of a significant fraction of the sulfhydryl (SH) groups under conditions compatible with the stability of the M_1 mAChR. Preliminary experiments showed that BrACh reacts efficiently and stoichiometrically with Cys SH groups under these conditions with a rate constant of approximately 35 $M^{-1}min^{-1}$ (Page, 1995), a figure which is similar to its rate of reaction with β-mercaptoethanol (Damle et al., 1978).

BrACh undergoes hydrolysis to bromoacetate and choline in aqueous solution. To try to assess the effect of this, the time course of BrACh inhibition of the binding of [^{3}H]NMS to wild-type M_1 mAChRs was measured at pH 8.0, and a comparison was made with ACh (Fig. 3a). Binding of [^{3}H]NMS in the presence of ACh was near to equilibrium after 15 min. A 3-fold higher concentration of BrACh gave similar levels of inhibition at 10 to 15 min, but after an incipient plateau, the degree of inhibition by BrACh steadily diminished. Thus, 1 mM BrACh gave 90% inhibition of the binding of 0.3 nM [^{3}H]NMS at 15 min, but this was reduced to 25 to 40% at 2 h. The time-dependent reduction in potency is probably attributable to the hydrolysis of BrACh to bromoacetate and cho-

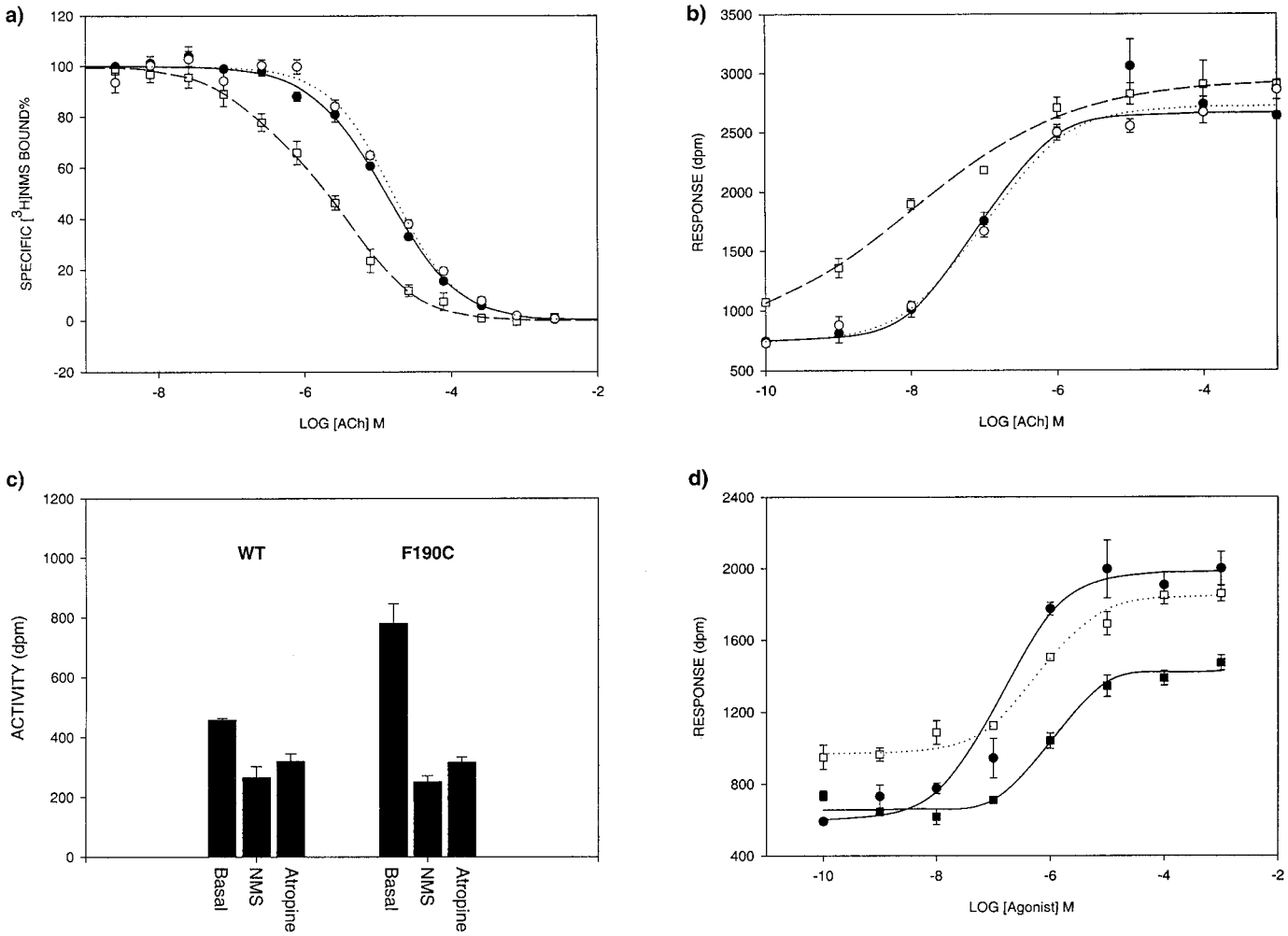

Fig. 2. Analysis of the Phe-190 → Cys mutant. Data are from representative experiments, which were repeated two to three times with the same results. Each point represents the mean ± S.E. of quadruplicate measurements. a, ACh inhibition of the binding of [^{3}H]NMS to wild-type (●), Phe-190 → Cys (□), and Phe-190 → Ala (○) M_1 mAChRs. Data have been corrected for the Cheng-Prusoff shift. Lines are fits to the Hill equation. Analysis of the wild-type and Phe-190 → Cys mutant using a two-site model of binding gave pK_d values of 4.7 and 5.2 for the low affinity sites and 5.7 and 6.7 for the high affinity sites, respectively. High affinity sites represented 40% of the total binding sites. b, PI dose-response curves for ACh potency at the wild-type (●), Phe-190 → Cys (□), and Phe-190 → Ala (○) M_1 mAChRs. c, antagonist inhibition of basal signaling. Atropine or NMS (10^{-6} M) were added to COS-7 cells expressing wild-type and Phe-190 → Cys receptors 24 h before assay. Cells were washed three times with Krebs-Henseleit solution to remove residual antagonist before addition of Krebs-Henseleit solution containing 10 mM LiCl to initiate the PI assay. d, pilocarpine dose-response curves for the wild-type and Phe-190 → Cys mutant receptors. (●) ACh, wild-type; (■) pilocarpine, wild-type; (□) pilocarpine, Phe-190 → Cys.

line. Choline has an affinity of 10^3 M^{-1} for the M_1 mAChR (Page, 1995), and, in agreement with this, 1 mM choline caused a 25% inhibition of the binding of 0.3 nM [^{3}H]NMS. In contrast, bromoacetate failed to inhibit [^{3}H]NMS binding at concentrations up to 10 mM (data not shown).

To obtain a crude estimate of the apparent reversible affinity of BrACh for the wild-type and mutant receptors, the inhibition of the binding of [^{3}H]NMS (0.3 nM) was measured after a 15-min incubation. The corrected pIC_{50} for the wild-type receptor was 4.22, assuming competitive behavior with [^{3}H]NMS, and this appeared similar for all of the mutant receptors with the exception of Phe-190 → Cys, where the apparent affinity was 3-fold higher (Table 3).

The time-dependent hydrolysis of BrACh at pH 8.0 and 30°C to relatively inactive products was exploited to screen for irreversible reaction, by measuring the time-dependent reversal of the inhibition of [^{3}H]NMS binding. Most of the mutants (illustrated by the Met-194 → Cys mutant, Fig. 3b) behaved like the wild-type receptor. In contrast, the binding of [^{3}H]NMS to the Thr-192 → Cys mutant did not increase between 15 min and 2 h in the presence of 1 mM BrACh (Fig. 3b). The Ala-193 → Cys mutant showed intermediate behavior.

To examine the concentration dependence of BrACh blockade of Thr-192 → Cys receptors, membrane preparations were incubated with different concentrations of BrACh using a concentration of the membrane fraction 10 times that normally used in binding assays. After 15 min, the membranes were rapidly cooled to 0°C, diluted 10-fold with ice-cold binding buffer, and incubated for 2 h at 30°C with a receptor-saturating concentration of [^{3}H]NMS to allow hydrolysis of BrACh and subsequent binding of [^{3}H]NMS. Wild-type and Thr-192 → Ala receptors were used as comparisons in this experiment. Controls were incubated without BrACh. The results are summarized in Fig. 4.

The block of the Thr-192 → Cys mutant by BrACh increased from 0.03 mM and reached a plateau at 1 mM, at which point approximately 50% of the [^{3}H]NMS binding sites were occluded. The BrACh concentration causing 50% of the maximum effect was between 0.1 and 0.3 mM. Calculation of the apparent rate constant of reaction of BrACh with the Cys-192 mutant as outlined by Javitch et al. (1995) and Marjamäki et al. (1998) gave a value of 150 $M^{-1}min^{-1}$. This value is not corrected for the hydrolysis of BrACh. A concentration of 3 mM BrACh was necessary to give more than 20% inhibition of [^{3}H]NMS binding to the wild-type receptor or the Thr-192 → Ala mutant.

In an attempt to increase the extent of the block of Thr-192 → Cys, two successive additions of 1 mM BrACh were made, each being incubated for 15 min. Blockade increased to 72% with little additional effect on the wild-type receptor (data not shown). Thus, the main factor limiting the rate and extent of block is likely to be the relatively rapid hydrolysis of BrACh at pH 8.0.

Saturation binding curves were measured for membranes that had been pretreated with 1 mM BrACh for 15 min, diluted 20-fold with ice-cold binding buffer, centrifuged to

TABLE 2

Binding of formylcholine to wild-type and TMD 5 mutant M_1 mAChRs

Formylcholine was dried to constant weight in a SpeedVac before use. To minimize the hydrolysis of FCh, competition binding assays were performed for 30 min at pH 7.5. Data were analyzed using the Hill equation to generate an IC_{50} value and a Hill coefficient, n_H. Affinities have been corrected for the Cheng-Prusoff shift. Fold-effect is the decrease in FCh binding affinity compared with the wild-type level. Results are the mean ± range or S.E. of n determinations.

Mutant	pIC_{50}	n_H	Fold Effect	n
	−log M			
WT	3.39 ± 0.04	0.99 ± 0.02		5
I188C	2.96 ± 0.10*	1.00 ± 0.13	2.7	3
I188A	3.21 ± 0.07	0.95 ± 0.06	1.5	2
T192C	3.12 ± 0.08*	0.94 ± 0.06	1.9	3
T192A	3.22 ± 0.07	0.96 ± 0.13	1.5	2
A196C	2.93 ± 0.06*	1.01 ± 0.05	2.9	3
A196G	3.35 ± 0.18	0.89 ± 0.09	1.1	2

* $P < .01$ with respect to wild-type control.

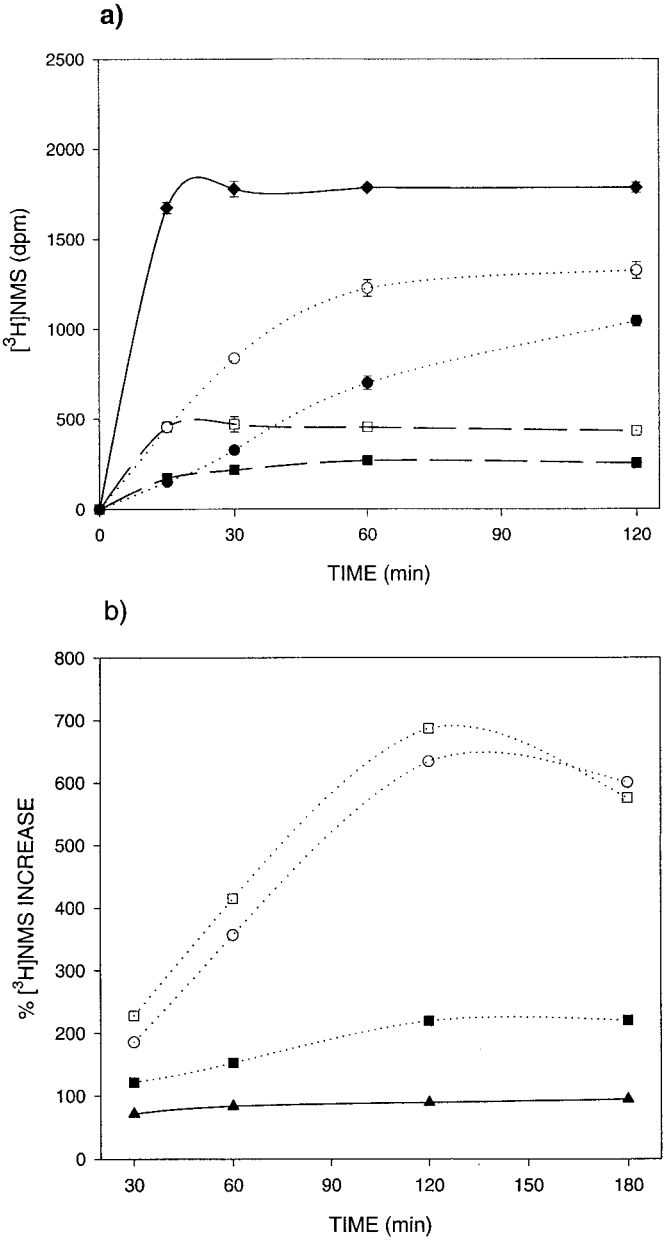

Fig. 3. Time course of inhibition of [^{3}H]NMS binding by ACh and BrACh at wild-type and TMD 5 mutant M_1 mAChRs. a, time course of binding of [^{3}H]NMS (0.3 nM) to wild-type M_1 mAChRs in the absence (♦) and presence of ACh (□, 0.1 mM; ■, 0.3 mM) and BrACh, (○, 0.3 mM; ●, 1 mM). Measurements were made at pH 8.0 and 30°C. b, time course of binding of [^{3}H]NMS (0.3 nM) to Thr-192 → Cys (▲), Ala-193 → Cys (■), Met-194 Cys (□), and wild-type (○) M_1 mAChRs in the presence of BrACh (1 mM).

remove excess BrACh, and resuspended. The affinity of [^{3}H]NMS for wild-type or Thr-192 → Cys receptors was unaltered by pretreatment with BrACh. Although the B_{max} value of the wild-type membranes was not significantly affected, the B_{max} value of the Thr-192 → Cys membranes was reduced by 70 ± 14% (n = 2).

To assess ligand protection of the blockade reaction, Thr-192 → Cys membranes were pretreated for 10 min with a range of concentrations of NMS (0.1–100 nM) before exposure to BrACh (1 mM) for 15 min, and assay of the extent of blockade by incubation with a receptor-saturating concentration of [^{3}H]NMS for 2 h. NMS pretreatment reduced the extent of BrACh block from 48 ± 2% to 12 ± 0.2% (75% protection), with an IC_{50} of 1 nM, a value consistent with the establishment of a reversible competitive interaction between NMS and BrACh before reaction. However, NMS, at concentrations up to 10 nM, did not appreciably affect the small degree of inhibition of Ala-193 → Cys by BrACh. This may reflect the reduced affinity of this mutant for NMS (Table 1), which may lead to less effective competition.

A concentration of 1 mM BrACh was used to quantitate the relative reactivities of the different mutants. This was sufficient to give an initial reversible occupancy in excess of 90% in each case (Table 3). A 10-min incubation was used to attempt to restrict the reaction to the initial part of the blockade time course. The results are summarized in Fig. 5.

A 10-min incubation led to 37% block of the binding of [^{3}H]NMS to Thr-192 → Cys and 23% block of Ala-193 → Cys. Blockade of the wild-type receptor, and of the other Cys substitution mutants was about 10%.

The introduction of bulky substituents into the side chain of ACh is known to reduce its efficacy. It was therefore of interest to investigate the properties of BrACh as an agonist. BrACh acted as a weak partial agonist with respect to the PI response elicited by the wild-type M_1 mAChR expressed in COS-7 cells (Fig. 6), with an EC_{50} value of 50 μM, and a maximum response of 30% of the wild-type value. BrACh did not activate the Thr-192 → Cys mutant (Fig. 6). Concentrations of BrACh higher than 3 mM caused nonspecific activation of the PI pathway in COS-7 cells (data not shown). Experiments in which cells expressing wild-type and mutant receptors were preincubated with BrACh have so far failed to give any evidence for irreversible stimulation of the PI signal.

Methanethiosulfonate Reagents and Iodoacetamide. Additional studies were made using methanethiosulfonate reagents, which have previously been applied to investigations of Cys substitution mutants of the D_2 dopamine receptor (Javitch et al., 1995) and the α_2-adrenergic receptor (Marjamäki et al., 1998, 1999). In addition, experiments were performed with the class-specific reagent, iodoacetamide.

Iodoacetamide failed to block the binding of [^{3}H]NMS to

TABLE 3

Apparent affinity of BrACh for wild-type and TMD 5 mutant M_1 mAChRs

Competition binding assays were performed at pH 8.0 for 15 min. Data were analyzed using the Hill equation. Values have been corrected for the Cheng-Prusoff shift. Results are the mean ± range or S.E. of n determinations.

Mutant	pIC_{50}	n_H	n
	−log M		
WT	4.22 ± 0.05	1.13 ± 0.05	7
I188C	4.15 ± 0.11	0.90 ± 0.09	3
I188A	4.04 ± 0.11	1.09 ± 0.07	3
T189S	4.15 ± 0.12	1.06 ± 0.09	3
T189C	4.14 ± 0.09	1.02 ± 0.15	2
F190C	4.87 ± 0.13	0.99 ± 0.11	2
F190A	4.10 ± 0.03	0.96 ± 0.16	3
G191C	4.23 ± 0.11	1.16 ± 0.05	3
G191A	4.58 ± 0.01	1.07 ± 0.06	2
T192C	4.30 ± 0.04	1.03 ± 0.01	2
T192A	4.51 ± 0.02	0.82 ± 0.26	2
A193C	4.15 ± 0.10	1.09 ± 0.09	3
A193G	3.90 ± 0.12	1.03 ± 0.27	2
M194C	4.21 ± 0.20	1.26 ± 0.10	3
A195C	4.34 ± 0.13	1.20 ± 0.11	3
A195G	4.34 ± 0.13	1.18 ± 0.15	2
A196C	4.08 ± 0.06	1.10 ± 0.08	3
A196G	4.13 ± 0.19	1.06 ± 0.01	2

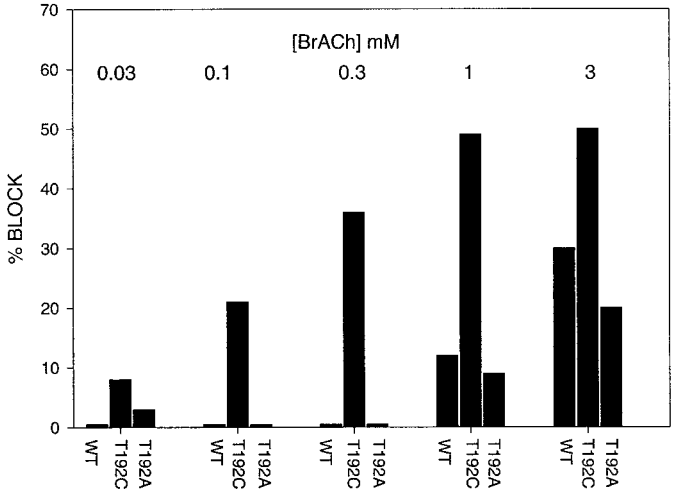

Fig. 4. Concentration dependence of blockade of wild-type, Thr-192 → Cys, and Thr-192 → Ala M_1 mAChRs by BrACh. Concentrated aliquots of receptors (10× final concentration) were preincubated with a range of BrACh concentrations for 15 min at 30°C. The concentrated membranes were diluted with ice-cold binding buffer (pH 8.0) containing [^{3}H]NMS (3 nM), and incubated for an additional 2 h at 30°C. Controls were treated in the same manner, without addition of BrACh.

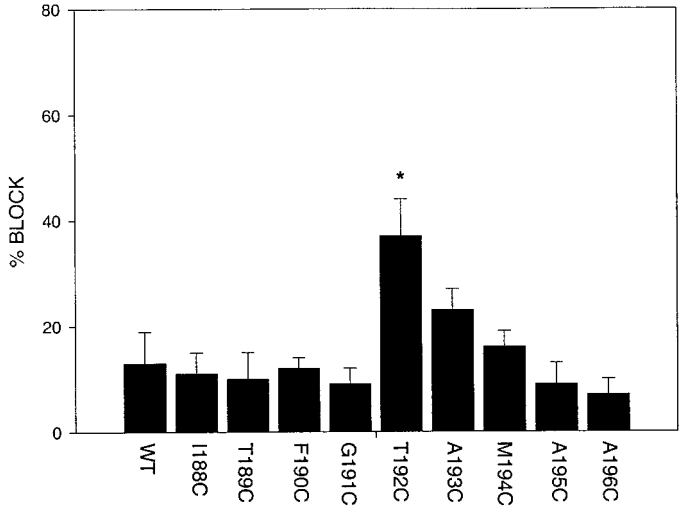

Fig. 5. Blockade of wild-type and TMD 5 mutant M_1 mAChRs by BrACh. Concentrated aliquots of membranes (10× final concentration) were preincubated with 1 mM BrACh for 10 min at 30°C. The concentrated membranes were diluted 10× with ice-cold binding buffer and incubated in the presence of 3 nM [^{3}H]NMS for 2 h at 30°C. Controls were treated in the same manner, without addition of BrACh. Percent block is calculated as the decrease in specific [^{3}H]NMS binding. Results are mean ± S.E. of three or more independent determinations. *P < .05 with respect to BrACh-treated wild-type receptor.

either the wild-type or the Thr-192 → Cys mutant receptor at concentrations up to 10 mM. Similarly, the negatively charged MTSES did not react with the wild-type receptor, or with the Thr-192 → Cys mutant. In contrast, the tertiary amine, MTSEA (0.1 mM) blocked the binding site of the wild-type receptor, and unlike BrACh, must react rapidly with a Cys residue intrinsic to the wild-type receptor. These reagents were not investigated further.

MTSET, which, like BrACh, has a quaternary ammonium headgroup, showed greater selectivity (Fig. 7). Using a 10-min incubation, 0.1 mM MTSET gave less than 20% block of the wild-type receptor but over 70% block of Thr-192 → Cys and Ala-193 → Cys. Unlike BrACh, it also showed evidence of reaction with Phe-190 → Cys but not with the other Cys mutants. This pattern was maintained at 0.3 and 1 mM MTSET (Fig. 7 and data not shown). Calculation of the apparent rate constants for blockade of the mutants by MTSET gave values of 670 $M^{-1}min^{-1}$ for Phe-190 → Cys and 1300 $M^{-1}min^{-1}$ for Thr-192 → Cys and Ala-193 → Cys.

Pretreatment of the Phe-190 → Cys, Thr-192 → Cys, and Ala-193 → Cys mutants with 10 mM iodoacetamide did not block their subsequent reaction with BrACh or MTSET. Pretreatment of the Phe-190 → Cys and Ala-193 → Cys mutants with 1 mM BrACh did not affect the subsequent block of [^{3}H]NMS binding by MTSET, provided that the free BrACh concentration was reduced first; this was achieved by pelleting the membranes and resuspending them. Similarly, in the case of the Thr-192 → Cys mutant, the [^{3}H]NMS binding sites that had not reacted with BrACh were blocked by subsequent reaction with MTSET. These experiments appear to exclude false negatives, in which iodoacetamide or BrACh react with particular mutants without affecting the binding of [^{3}H]NMS.

Pretreatment of the mutant receptors with NMS gave 90% protection of all three Cys mutant receptors against block by 0.1 mM MTSET, with IC_{50} values of approximately 0.3 nM (Thr-192 → Cys, Ala-193 → Cys) and 0.9 nM (Phe-190 → Cys). The differences in IC_{50} values for NMS protection are consistent with the values of the NMS affinity measured by direct saturation binding (Table 1).

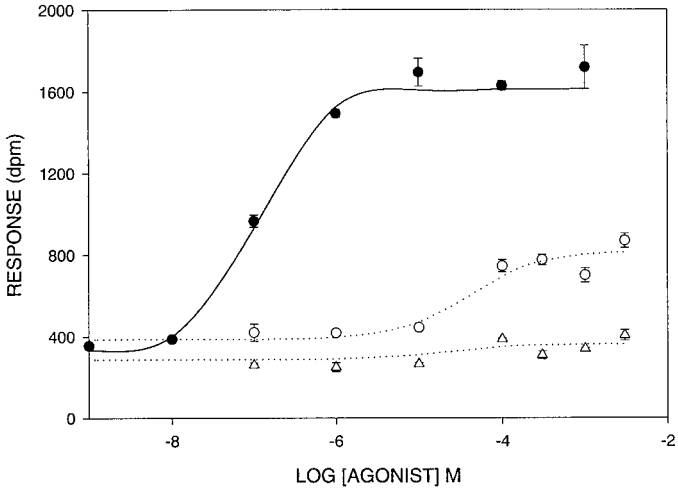

Fig. 6. Activation of wild-type and T192C M_1 mAChRs by BrACh. The PI response to BrACh was determined as described under *Experimental Procedures*, using a 30-min incubation. (●) ACh, wild-type; (○) BrACh, wild-type; (△) BrACh, Thr-192 → Cys. The pEC_{50} value for BrACh activation of the wild-type receptor was 4.38 ± 0.16 (n = 3), and the E_{max} value was 30 ± 6% of the wild-type value for ACh. Points are mean ± S.E. of triplicate measurements from a representative experiment.

Discussion

The stretch of nine residues in TMD 5 of the M_1 mAChR examined in this study contains the threonine residues postulated to hydrogen bond to the ACh side chain (Wess et al., 1991, 1992; Wess, 1996), and the alanine residues homologous to the catechol-binding serine residues in the catecholamine receptors. These residues have been replaced by cysteines, to allow reaction with sulfhydryl reagents, and by smaller amino acids, to probe the functional importance of each side chain.

The mutations had small effects on receptor expression levels, suggesting that none of the target amino acid side chains make intramolecular contacts crucial for the stability of the structure. The largest effect on the binding affinity of the antagonist [^{3}H]NMS was a 3-fold reduction resulting from mutation of Ala-193 (position 5.43) to Cys or Gly. Thus, blockade of [^{3}H]NMS binding could be used to monitor the reaction of the Cys-substituted mutants with methanethiosulfonate reagents.

Reactivity with these polar reagents has been used to define the accessibility of residues from within the aqueous binding cleft of the D_2 dopamine and the α_{2A} receptors (Javitch et al., 1995; Marjamäki et al., 1998). In the D_2 receptor, this required the substitution of Cys-118 in TMD 3, which otherwise reacts rapidly, blocking the binding site. There is no corresponding Cys in TMD 3 of the mAChR sequences. Accordingly, the quaternary ligand MTSET, and the negatively charged analog MTSES did not react rapidly with the binding site of the wild-type M_1 mAChR at concentrations up to 1 mM. The tertiary amine ligand MTSEA,

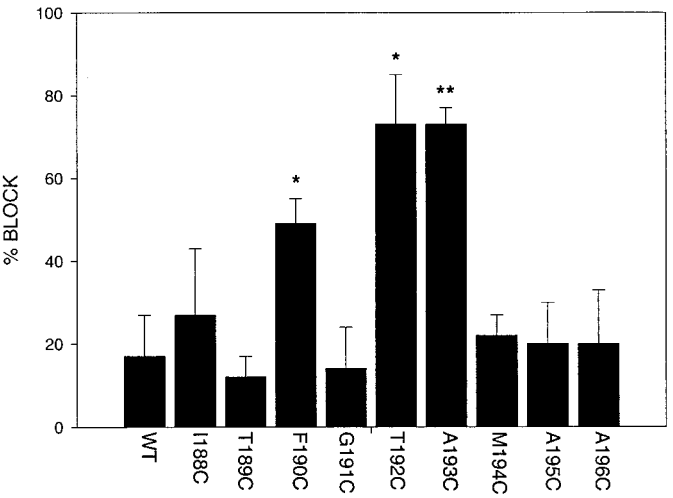

Fig. 7. Blockade of wild-type and TMD 5 mutant M_1 mAChRs by MTSET. Concentrated aliquots of membranes (10× final concentration) were preincubated with 0.1 mM MTSET for 10 min at 30°C. The concentrated membranes were diluted 10× with ice-cold binding buffer and incubated in the presence of 3 nM [^{3}H]NMS for 2 h at 30°C. Controls were treated in the same manner, without addition of MTSET. Percent block is calculated as the decrease in specific [^{3}H]NMS binding. Results are mean ± S.E. of three or more independent determinations. *$P < .05$; **$P < .01$ with respect to MTSET-treated wild-type receptor. Similar results were obtained at 0.3 mM MTSET: percent block values were wild-type, 30 ± 9%; Phe-190 → Cys, 65 ± 5%; Thr-192 → Cys, 91 ± 11%; Ala-193 → Cys, 94 ± 6%. Other mutants were similar to wild-type.

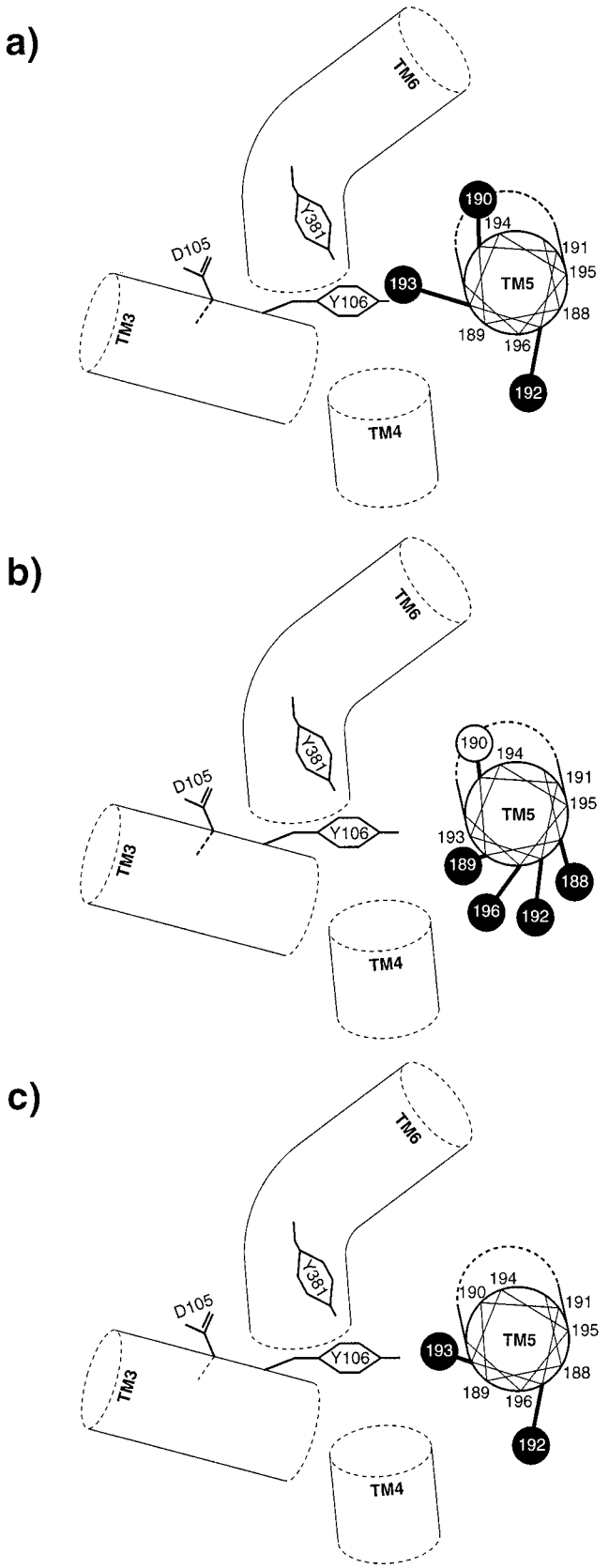

Fig. 8. Results of TMD 5 mutations in the context of the Baldwin model of the transmembrane domain of the 7-transmembrane receptors. The exofacial regions of TMDs 3, 4, 5, and 6 are represented. Key residues in the binding of the ACh headgroup are Asp-105 and Tyr-106 in TMD 3, and Tyr-381 in TMD 6. In these drawings, Thr-192 and Ala-193 occur at approximately the same depth as Asp-105, Tyr-106, and Tyr-381, whereas Ile-188, Thr-189, and Phe-190 are found one helical turn above and Ala-196 is found one helical turn below this level. a, MTSET blockade. The positions of the side chains of the Cys residues that gave accentuated block are shown. The lengths of the lines are proportional to the extent of block by 0.1 mM MTSET. b, effect on ACh affinity. The positions of the side chains whose mutation to Cys affected ACh affinity are shown. The lengths of the lines are proportional to the magnitudes of the effect. Positions 188, 189, 192, and 196 gave decreased affinity. Position 190 gave increased affinity. c, BrACh blockade. The lengths of the lines are proportional to the extent of block by 1 mM BrACh.

however, blocked the wild-type binding site, precluding its use.

MTSET has a quaternary ammonium headgroup potentially capable of binding to Asp-105, Tyr-106 (TMD 3), and Tyr-381 (TMD 6), which are thought to be important for ligating ACh itself (Spalding et al., 1994; Page et al., 1995; Hulme et al., 1999; Lu and Hulme, 1999; Ward et al., 1999). This may explain its highly selective blockade of the Cys-substituted M_1 mAChR mutants. MTSET reacted strongly with mutant receptors in which Cys residues were substituted for Thr-192 (position 5.42) and Ala-193 (position 5.43). In the Baldwin model of the transmembrane region of the 7-transmembrane receptors (Baldwin et al., 1997), these residues are positioned at the same level in the receptor structure as Asp-105, Tyr-106, and Tyr-381. A third site, Phe-190 (position 5.40), which showed a slightly lower susceptibility, occurs one helical turn higher up. The effective protection of these residues against reaction with MTSET by the antagonist NMS suggests that they form part of the face of TMD 5, which is accessible from the binding cleft in the ground state of the receptor. This agrees with the orientation of TMD 5 proposed in the Baldwin model (Fig. 8a). These findings resemble the observations of Marjamäki et al., (1999) on the α_2-adrenergic receptor, but differ from the pattern of reactivity found in the Cys-118 → Ser D_2 receptor (Javitch et al., 1995), which suggested a strand rather than a helical conformation for the homologous sequence.

Mutations of four amino acids, Ile-188, Thr-189, Thr-192, and Ala-196 (positions 5.38, 5.39, 5.42, and 5.46) significantly reduced both the affinity of ACh and the potency of ACh in activating the receptor. The effects of mutating the residues to Cys, or to Ala or Gly, in the case of Ile-188, Thr-192, and Ala-196, were similar and the effects on binding and activity were also of similar magnitudes.

A calculation of the signal transduction efficacies of the mutants (Whaley et al., 1994; Lu and Hulme, 1999), taking into account the variations in receptor expression levels, indicated greater than 3-fold reductions in efficacy for PI signaling for Thr-192 → Ala and Ala-196 → Gly, but no significant change for the corresponding Cys mutations (Fig. 1). These residues therefore make a contribution to ACh affinity and efficacy. However, they may not be as important for transducing ACh binding into receptor activation as are certain other residues, for example, Tyr-106 (Lu and Hulme, 1999) and Tyr-381 (Ward et al., 1999). Interpreted in terms of a two-state model of receptor binding, Thr-192 and Ala-196 may make interactions that help to anchor ACh in the binding site in the ground state of the receptor, which then undergo a modest enhancement in the activated state (Hulme et al., 1999). The effect on PI signaling efficacy was smaller than that reported for the homologous mutations in the M_3 mAChR (Wess et al., 1992).

The Thr-192 → Ala mutation has also been studied after expression in A9L cells (Huang et al., 1999). Relatively modest reductions in the low-affinity binding of ACh and carbachol and of the potency and efficacy of carbachol in PI signaling were reported. Interestingly, in contrast to the situation found in COS-7 cells, a significant fraction of GTP-sensitive high-affinity binding sites was detected, and these showed greater sensitivity to the effects of the Thr-192 → Ala mutation. The contribution of Thr-192 to agonist binding may depend on the nature of the G-protein coupled to the receptor.

The mutations of Ile-188, Thr-189, Thr-192, and Ala-196 had a similar but smaller effect on the binding of two other agonists, carbachol and oxotremorine-M. Their effects on the binding of formylcholine were very small. Because formylcholine lacks the terminal methyl group of ACh, this seems consistent with a contribution by these residues to a methyl-binding pocket, which is important for anchoring the extremity of the side chain of ACh and closely related compounds. In contrast, the adjoining 5.43 position is important for activation of the D_{2S} receptor by dopamine (Wiens et al., 1998), but not the M_1 mAChR by ACh.

Represented on a polar plot (Fig. 8b), all of the residues whose mutation decreased ACh affinity are clustered within a single 90° sector, further supporting a helical conformation for this part of TMD 5. Positions 5.38, 5.42, and 5.46, whose mutation most inhibited ACh binding, are identical with those that perturbed the binding of the antagonist sulpiride to the D_2 receptor (Javitch et al., 1995). In the context of the Baldwin model, the center of this patch of residues is offset to one side of the binding cleft, appearing skewed toward the lipid bilayer beyond TMD 4. Interestingly, position 5.40, whose mutation to Cys increased receptor signaling efficacy, and induced basal activity, is modeled at the other side of the binding cleft, at the interface with TMD 6. Rotations of TMD 6 are implicated in receptor activation (Gether et al., 1997; Javitch et al., 1997; Spalding et al., 1998), and could be modified by the introduction of a polarizable SH group into the neighboring helix.

To establish more directly the potential of residues in this sequence to contact muscarinic ligands, we probed the Cys substitution mutants with the alkylating ACh analog, BrACh. BrACh was less reactive than MTSET. Nevertheless, it was even more selective, reacting primarily with Thr-192 → Cys (position 5.42), less with A1a-93 → Cys (position 5.43), and not with other sequence positions, according to blockade of [^{3}H]NMS binding (Fig. 8c). It is interesting that a Cys residue introduced at position 5.42 in the α_2-receptor was selectively modified by chloroethylclonidine relative to MT-SEA (Marjamäki et al., 1999).

The rate constant estimated for the BrACh reaction with Thr-192 → Cys (greater than 150 $M^{-1}s^{-1}$) was slower than that reported for its reaction with the reduced nicotinic receptor binding site (5000 $M^{-1}s^{-1}$; Damle et al. 1978). This may reflect the existence of alternative binding modes for the ligand, which become favored in the mutant receptor because of disruption of the primary binding interaction, as proposed previously for the binding of ACh analogs (Page et al., 1995). This could have the effect of reducing the concentration of the reactive species, contributing to the lower rate of reaction with the mutated mAChR binding site relative to the nicotinic binding site, in which the primary binding interactions may be less affected by reduction of the disulfide bond.

Specific binding interactions between the ester moiety of ACh side chain and hydrogen bonding moieties, such as the hydroxyl group of Tyr-381 (Ward et al., 1999), may restrict the mobility of the side chain of BrACh within the binding site of the mutant receptor. These interactions are not available to MTSET. Both the selectivity of BrACh for the Thr-192 → Cys mutant and the attenuation of the effect of the Thr-192 → Ala and Thr-192 → Cys mutations by the alteration (carbachol) or deletion (formylcholine) of the terminal methyl group of ACh suggest that interactions between the ester methyl group of ACh and the methyl group of Thr-192 make a significant contribution to the binding of ACh. Neighboring residues such as Ile-188, Thr-189, Ala-193, and Ala-196 may then complete a binding pocket for the ACh methyl group. The asymmetric location of this pocket with respect to the center of the accessible face of TMD 5 suggests the possibility that a rotation of TMD 5 may be needed to optimize the binding of ACh. An ACh-induced rotation of TMD 5 could contribute to the formation of the activated state of the receptor.

References

Allman K, Page KM and Hulme EC (1997) Cysteine scanning mutagenesis of transmembrane domain V of the m1 muscarinic receptor. *Life Sci* **60:**1177.

Baldwin JM, Schertler GFX and Unger VM (1997) An alpha-carbon template for the transmembrane helices in the rhodopsin family of G-protein-coupled receptors. *J Mol Biol* **272:**144–164.

Ballesteros JA and Weinstein H (1995) Integrated methods for the construction of three dimensional models and computational probing of structure-function relations in G-protein coupled receptors. *Methods Neurosci* **25:**366–428.

Boess FG, Monsma FJ, Myer V, Zwingelstein C and Sleight AJ (1997) Interaction of tryptamine and ergoline compounds with threonine 196 in the ligand binding site of the 5-hydroxytryptamine$_6$ receptor. *Mol Pharmacol* **52:**515–523.

Cavalli A, Fanelli F, Taddei C, De Benedetti PG and Cotecchia S (1996) Amino acids of the α_{1B}-adrenergic receptor involved in agonist binding: Differences in docking catecholamines to receptor subtypes. *FEBS Lett* **399:**9–13.

Colquhoun D (1998) Binding, gating, affinity and efficacy: The interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. *Br J Pharmacol* **125:**924–947.

Damle VN, McLaughlin M and Karlin A (1978) Bromoacetylcholine as an affinity label of the acetylcholine receptor from *Torpedo californica*. *Biochem Biophys Res Commun* **84:**845–851.

Gantz I, DelValle J, Wang L, Tashiro T, Munzert G, Guo Y-J, Konda Y and Yamada T (1992) Molecular basis for the interaction of histamine with the histamine H_2 receptor. *J Biol Chem* **267:**20840–20843.

Gether U, Lin S, Ghanouni P, Ballesteros JA, Weinstein H and Kobilka BK (1997) Agonists induce conformational changes in transmembrane domains 3 and 6 of the β_2-adrenoceptor. *EMBO J* **16:**6737–6747.

Ho BY, Karschin A, Branchek T, Davidson N and Lester HA (1992) The role of conserved aspartate and serine residues in ligand binding and in function of the 5-HT$_{1A}$ receptor: A site-directed mutation study. *FEBS Lett* **312:**259–262.

Huang X-P, Nagy PI, Williams FE, Peseckis SM and Messer WS (1999) Roles of threonine 192 and asparagine 382 in agonist and antagonist interactions with M_1 muscarinic receptors. *Br J Pharmacol* **126:**735–745.

Hulme EC and Lu Z-L (1998) Scanning mutagenesis of transmembrane domain 3 of the M_1 muscarinic acetylcholine receptor. *J Physiol (Paris)* **92:**269–274.

Hulme EC, Lu Z-L, Ward SDC, Allman K and Curtis CAM (1999) The conformational switch in 7-transmembrane receptors: The muscarinic receptor paradigm. *Eur J Pharmacol* **375:**247–260.

Hwa J and Perez DM (1996) The unique nature of the serine interactions for α_1-adrenergic receptor agonist binding and activation. *J Biol Chem* **271:**6322–6327.

Javitch JA, Fu D and Chen J (1995) Residues in the fifth membrane-spanning segment of the dopamine D2 receptor exposed in the binding-site crevice. *Biochemistry* **34:**16433–16439.

Javitch JA, Fu D, Liapakis G and Chen J (1997) Constitutive activation of the β_2 adrenergic receptor alters the orientation of its sixth membrane-spanning segment. *J Biol Chem* **272:**18546–18549.

Jones PG, Curtis CA and Hulme EC (1995) The function of a highly-conserved arginine residue in activation of the muscarinic M_1 receptor. *Eur J Pharmacol* **288:**251–257.

Lu Z-L, Curtis CAM, Jones PG, Pavia J and Hulme EC (1997) The role of the aspartate-arginine-tyrosine triad in the m1 muscarinic receptor: Mutations of aspartate 122 and tyrosine 124 decrease receptor expression but do not abolish signaling. *Mol Pharmacol* **51:**234–241.

Lu Z-L and Hulme EC (1999) The functional topography of transmembrane domain

3 of the M_1 muscarinic acetylcholine receptor, revealed by scanning mutagenesis. *J Biol Chem* **274:**7309–7315.
Mansour A, Meng F, Meador-Woodruff JH, Taylor LP, Civelli O and Akil H (1992) Site-directed mutagenesis of the human dopamine D_2 receptor. *Eur J Pharmacol* **227:**205–214.
Marjamäki A, Frang H, Pihlavisto M, Hoffren A-M, Salminen T, Johnson MS, Kallio J, Javitch JA and Scheinin M (1999) Chloroethylclonidine and 2-aminoethylmethanethiosulfonate recognize two different conformations of the human α_{2A}-adrenergic receptor. *J Biol Chem* **274:**21867–21872.
Marjamäki A, Pihlavisto M, Cockcroft V, Heinonen P, Savola J and Scheinin M (1998) Chloroethylclonidine binds irreversibly to exposed cysteines in the fifth membrane-spanning domain of the human α_{2A}-adrenergic receptor. *Mol Pharmacol* **53:**370–376.
Ohta K, Hayashi H, Mizuguchi H, Kagamiyama H, Fujimoto K and Fukui H (1999) Site-directed mutagenesis of the histamine H-1 receptor-roles of aspartic acid 107, asparagine 198 and threonine 194. *Biochem Biophys Res Commun* **203:**1096–1101.
Page KM (1995) *The Molecular Basis of Agonist Action at Muscarinic Acetylcholine Receptors*, Ph.D. thesis, University of London, United Kingdom.
Page KM, Curtis CAM, Jones PG and Hulme EC (1995) The functional role of the binding site aspartate in muscarinic acetylcholine receptors, probed by site-directed mutagenesis. *Eur J Pharmacol* **289:**429–437.
Pollock NJ, Manelli AM, Hutchins CW, Steffey ME, MacKenzie RG and Frail DE (1992) Serine mutations in transmembrane V of the dopamine D_1 receptor affect ligand interactions and receptor activation. *J Biol Chem* **267:**17780–17786.
Sartania N and Strange PG (1999) Role of conserved serine residues in the interaction of agonists with D3 dopamine receptors. *J Neurochem* **72:**2621–2624.
Shortle D (1992) Mutational studies of protein structures and their stabilities. *Q Rev Biophys* **25:**205–250.
Spalding TA, Birdsall NJ, Curtis CA and Hulme EC (1994) Acetylcholine mustard labels the binding site aspartate in muscarinic acetylcholine receptors. *J Biol Chem* **269:**4092–4097.
Spalding TA, Burstein ES, Henderson SC, Ducote KR and Brann MR (1998) Identification of a ligand-dependent switch within a muscarinic receptor. *J Biol Chem* **273:**21563–21568.
Strader CD, Candelore MR, Hill WS, Sigal IS and Dixon RAF (1989) Identification of two serine residues involved in agonist activation of the β-adrenergic receptor. *J Biol Chem* **264:**13572–13578.
Strange PG (1996) The energetics of ligand binding at catecholamine receptors. *Trends Phamacol Sci* **17:**238–244.
Wang C-D, Buck MA and Fraser CM (1991) Site-directed mutagenesis of α_{2A}-adrenergic receptors: Identification of amino acids involved in ligand binding and receptor activation by agonists. *Mol Pharmacol* **40:**168–179.
Ward SDC, Curtis CAM and Hulme EC (1999) Alanine-scanning mutagenesis of transmembrane domain 6 of the M_1 muscarinic acetylcholine receptor suggests that Tyr^{381} plays key roles in receptor function. *Mol Pharmacol* **56:**1031–1041.
Wess J (1996) Molecular biology of muscarinic acetylcholine receptors. *Crit Rev Neurobiol* **10:**69–99.
Wess J, Gdula D and Brann MR (1991) Site-directed mutagenesis of the m3 muscarinic receptor: Identification of a series of threonine and tyrosine residues involved in agonist but not antagonist binding. *EMBO J* **10:**3729–3734.
Wess J, Maggio R, Palmer JR and Vogel Z (1992) Role of conserved threonine and tyrosine residues in acetylcholine binding and muscarinic receptor activation. *J Biol Chem* **267:**19313–19319.
Wetzel JM, Salon JA, Tamm JA, Forray C, Nakanishi H, Cui W, Vaysse PJ-J, Chiu G, Weinshank RL, Hartig PR, Branchek TA and Gluchowski C (1996) Modeling and mutagenesis of the human α_{1A}-adrenoceptor: Orientation and function of transmembrane helix V sidechains. *Receptors Channels* **4:**165–177.
Whaley BS, Yuan N, Birnbaumer L, Clark RB and Barber R (1994) Differential expression of the α-adrenergic receptor modifies agonist stimulation of adenylyl cyclase: A quantitative evaluation. *Mol Pharmacol* **45:**481–489.
Wiens BL, Nelson CS and Neve KA (1998) Contribution of serine residues to constitutive and agonist-induced signaling via the D_{2S} dopamine receptor: Evidence for multiple, agonist-specific active conformations. *Mol Pharmacol* **54:**435–444.

Send reprint requests to: Dr. E. C. Hulme, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom. E-mail: ehulme@nimr.mrc.ac.uk