Biochemical Pharmacology, Vol. 51, pp. 539–544, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02234-1

Constitutive Activation of Chimeric m2/m5 Muscarinic Receptors and Delineation of G-Protein Coupling Selectivity Domains

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ABSTRACT. To derive structure/function relationships for muscarinic receptor/G-protein coupling, the m2 and m5 muscarinic receptors and a series of m2/m5 chimeras were tested for agonist binding and functional responses in a cellular proliferation/transformation assay. m5, which mediates stimulation of phosphatidylinositol turnover, displayed robust activity in the proliferation assay, whereas m2, which mediates inhibition of adenylyl cyclase, was inactive in the proliferation assay. Chimeras that contained m2 sequences in the i2 or i3 loops had impaired activity or were inactive, respectively. Chimeras that contained m2 segments reaching from the N-terminus to TM2, or from TM6 to the C-terminus, had enhanced activity relative to m5, and a chimera with both of these elements was constitutively activated. BIOCHEM PHARMACOL 51;4:539–544, 1996.

KEY WORDS. receptor; G-protein; chimera; constitutive activity; signal transduction; structure/function

Muscarinic acetylcholine receptors are members of a large family of receptors that mediate signal transduction by coupling with G-proteins. Most members of this family display significant sequence homology, particularly within regions predicted to form TM 1–7)§ [1, 2]. Muscarinic receptors consist of five genetically defined subtypes (m1–m5) [3–6] that can be divided into at least two functional classes. m1, m3 and m5 couple to pertussis-toxin insensitive G-proteins to potently stimulate phospholipase C, promote calcium release from cytosolic stores, modulate ion channels, stimulate mitogenesis, and transform NIH 3T3 cells. m2 and m4 couple to pertussis-toxin-sensitive G-proteins to weakly stimulate phospholipase C, open inwardly rectifying potassium channels, and inhibit adenylyl cyclase. The m2 and m4 subtypes do not stimulate mitogenesis nor do they transform NIH 3T3 cells [7, 8], although they are mitogenic in other cell types [9].

We developed a convenient assay of receptor activity, using the reporter gene β-galactosidase, called R-SAT (patents pending, [10]). We have found that only phosphatidylinositol coupled receptors mediate robust responses in R-SAT, whereas receptors coupled to adenylyl cyclase have very low activity in this assay (see Ref. 10). We previously used R-SAT to demonstrate that m1, m3, and m5, but not m2 receptors couple to the G-protein G_q [11]. In the present study, we examined the structural requirements for signal transduction by testing a series of m2/m5 chimeric constructs for activity in R-SAT. By systematically replacing m5 domains with the corresponding m2 sequences, it was possible to map the domains coupled to proliferative signals in NIH 3T3 cells. We found that it was possible to identify critical signaling domains in this manner, and that multiple epitopes contribute to efficient coupling. Furthermore, one of the chimeric constructs tested was constitutively activated.

MATERIALS AND METHODS

Cell Culture

NIH 3T3 cells (ATCC No. CRL 1658) and COS7 cells were incubated at 37° in a humidified atmosphere (5% CO_2) in Dulbecco's Modified Eagle's Medium supplemented with 4500 mg/L glucose, 4 nM L-glutamine, 50 U/mL penicillin G, 50 U/mL streptomycin (A.B.I.) and 10% calf serum for 3T3 cells or 10% fetal bovine serum for COS7 cells (Gibco).

Transfection Procedure and Functional Assays

R-SAT assays were performed as follows: Cells were plated one day before transfection using 1×10^6 cells in 10 mL of medium per 10-cm plate. Cells were transfected by calcium precipitation as described by Wigler *et al.* [12], using 5 μg of the human m5 receptor [6] in the pcD expression vector [13], 5 μg pSV-β-galactosidase (Promega, Madison, WI) and 20 μg of salmon sperm DNA (Sigma, St. Louis, MO). One day after transfection medium was changed, and after 2 days cells were trypsinized and aliquoted into the wells of a 96-well plate (100

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Received 18 May 1995; accepted 22 September 1995.

§ *Abbreviations:* TM 1–7, transmembrane regions 1–7; il-3, intracellular loops 1–3; Ni3 and Ci3, N- and C-termini of intracellular loop 3; and R-SAT, Receptor Selection and Amplification Technology.

μL/well). One 10-cm plate yields enough cells for 96 wells. Ligands were combined with the cells to a final volume of 200 μL/well. After 5 days in culture β-galactosidase levels were measured essentially as described by Lim and Chae [14]. The medium was aspirated from the wells, and the cells were rinsed with PBS (pH 7.4). Two hundred microliters of PBS with 3.5 mM *o*-nitrophenyl-β-D-galactopyranoside and 0.5% Nonidet P-40 (both Sigma) was added to each well, and the 96-well plate was incubated at room temperature. After 16 hr the plates were read at 405 nm on a plate-reader (Bio-Tek EL 310 or Molecular Devices). Data from R-SAT assays were fitted to the equation:

$$R = D + (A - D)/(1 + (x/c))$$

where A = minimum response, D = maximum response and c = EC_{50} (R = response, x = concentration of ligand). Curves were generated by least-squares fits using the program KaleidaGraph™ (Abelbeck Software). The pharmacology of carbachol and several other muscarinic agonists and antagonists, as determined with R-SAT, was very similar to that determined with traditional functional assays and has been presented elsewhere [15].

Binding Studies

For all binding studies, receptors were transiently expressed in COS7 cells; cells were harvested 72 h after transfection and stored at −80°. Membranes were prepared in binding buffer containing 25 mM sodium phosphate (pH 7.4), 5 mM magnesium, and 50 μM GppNHp as described [16] immediately before use. Binding reactions were allowed to proceed at room temperature for 4 h. Reactions were terminated by filtration. Filters were washed with ice-cold buffer and dried, and bound radioactivity was counted. Nonspecific binding was assessed in the presence of 1 μM atropine. The IC_{50} values for carbachol were assessed in the presence of 500 pM [^{3}H]*N*-methylscopolamine (NMS) as described [16]. Data from binding experiments were fitted to:

$$y = B_{max} \cdot IC_{50}^{n}/(IC_{50}^{n} + [L]^{n})$$

where y = [^{3}H]NMS specifically bound, n = Hill number, and L = [^{3}H]NMS concentration, to obtain the IC_{50} and the total number of binding sites (B_{max}).

Construction of m2/m5 Chimers

The m2/m5 chimers have been described [17].

RESULTS

We have developed a convenient cellular assay of receptor activity, which is based on the ability of phosphatidylinositol-coupled muscarinic receptors to confer a proliferative advantage upon NIH 3T3 cells [18] and which uses reporter genes to monitor this receptor-mediated stimulatory effect [10]. As shown in Fig. 1, when β-galactosidase and m5 muscarinic acetylcholine receptor expression vectors were co-transfected into NIH 3T3 cells, there was a concentration-dependent amplification of cells cultured in the presence of carbachol. When the m2 receptor, which is coupled to inhibition of adenylyl cyclase, was substituted for m5, there was no response in R-SAT. By systematically replacing segments of m5 with the corresponding m2 sequences, we sought to map the domains of m5 coupled to amplification of NIH 3T3 cells.

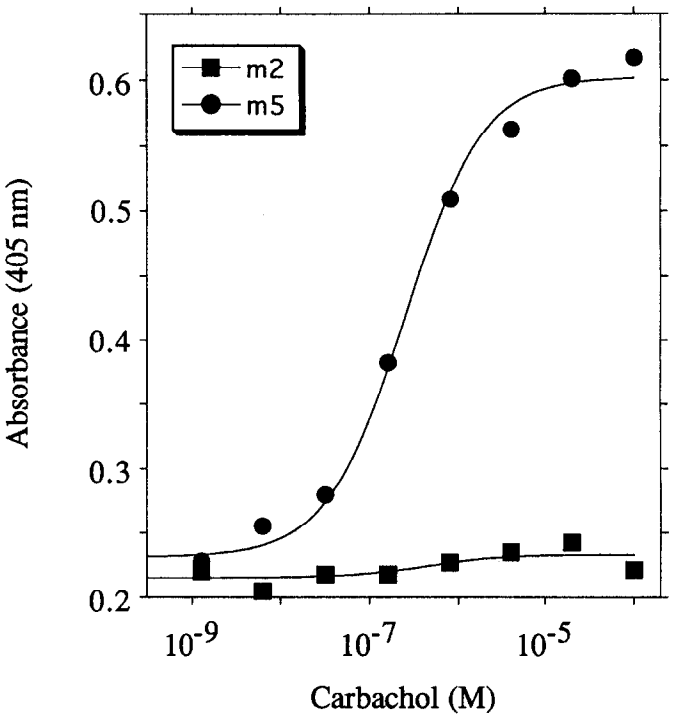

FIG. 1. R-SAT assay. m5 or m2 was co-transfected with β-galactosidase into NIH 3T3 cells, cultured in the presence of the indicated concentrations of carbachol, and induced β-galactosidase activity was assayed as described in Materials and Methods. Curve fits were performed as described in Materials and Methods.

To investigate the structural basis for the subtype differences in R-SAT, we tested a series of chimeric m2/m5 receptors (Fig. 2). All constructs were comparably expressed as assessed by their abilities to specifically bind the nonselective muscarinic antagonist [^{3}H]NMS [17].

We then tested the ability of each construct to amplify NIH 3T3 cells in R-SAT. As shown in Fig. 2, with only one exception, the chimeric constructs were all functional to varying degrees. Several of the chimeras were impaired relative to wild-type m5 (Fig. 3A, Table 1) including CR3, which was totally inactive, and CR2 and CR5, which were partially impaired. CR3 contains the m2 sequence spanning the N-terminus of the third intracellular loop (Ni3), while CR2 and CR5 both contain the m2 sequence spanning the second intracellular loop (i2). Surprisingly, several of the chimeras were hyperactive compared with m5. Carbachol was 10- and 2-fold more potent on CR1 and CR4, respectively, than on m5 (Fig. 3B and C, and Table 1). These chimeras contain the m2 sequence at the N-terminus and near the C-terminus, respec-

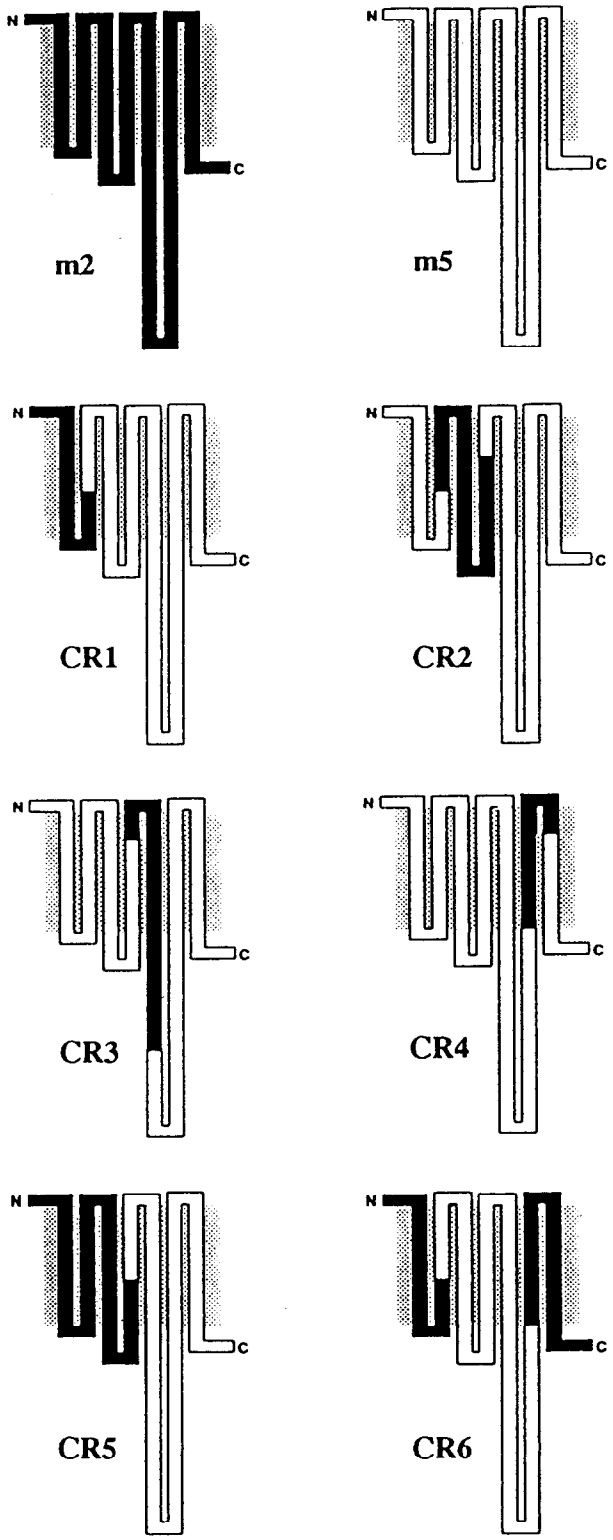

FIG. 2. Structure of the chimeric muscarinic receptors (CR1 to CR6) composed of human m2 (■) and human m5 (□) sequences (see Ref. 17). N and C depict the amino and carboxyl termini, located on the extracellular and cytoplasmic sides of the plasma membrane, respectively. The smaller length of wild-type m2 compared with m5 receptors (−7 amino acids in the N-terminus, −48 amino acids in the third cytoplasmic loop, −11 amino acids in the carboxyl-terminal tail) is ignored. The human m2 and m5 receptors are composed of 466 and 532 amino acids, respectively [5, 6]. The individual chimeras are composed as follows (amino acid numbers); CR1, m2 1–69/m5 77–532; CR2, m5 1–76/m2 70–155/m5 163–532; CR3, m5 1–162/m2 156–300/m5 336–532; CR4, m5 1–445/m2 391–421/m5 477–532; CR5, m2 1–155/m5 163–532; CR6, m2 1–69/m5 77–445/m2 391–466.

tively. CR6, which contains sequence elements found in both CR1 and CR4, was constitutively activated. Carbachol was 20-fold more potent at CR6 than on m5, and CR6 had a markedly elevated basal activity and increased maximal response relative to m5 (Fig. 3D and Table 1).

The constitutive activity of CR6 was reversed fully in a concentration-dependent manner by the potent muscarinic antagonist atropine, indicating that atropine is actually a negative antagonist (Fig. 4). The IC_{50} for atropine was very similar to the IC_{50} values determined for antagonism of the constitutive activity of m5 receptors activated by mutation [19] or by overexpression of the G-protein $G_{\alpha Q}$ [11]. Binding studies indicated that the affinity of carbachol for CR6 (6.0 ± 0.4 μM, Hill number = 0.57) was markedly higher than for m5 (22.4 ± 2.9 μM, Hill number = 0.65) and slightly higher than for m2 (6.4 ± 0.6 μM, Hill number = 0.68).

DISCUSSION

We have analyzed a series of chimeras between m2 and m5 which represent the two functional classes of muscarinic receptors. We observed two basic phenotypes: chimeras with diminished activity and chimeras with enhanced activity relative to wild-type m5. The chimeras with diminished activity all had, in common, m2 sequences in either the i2 or i3 loops. In particular, CR3, which had an "m2" Ni3 sequence, had no activity. These results agree strongly with previous results showing that the third cytoplasmic loop (i3) defines receptor subtype specificity for distinct G-proteins [20–23]. We were not able to test a chimera with m2 sequence spanning the Ci3 region because such chimeras fail to be expressed as functional receptors [17]. However, we recently completed an extensive analysis of the Ci3 regions of m5 using random saturation mutagenesis in which we found that Ci3 is critical determinant of receptor affinity for G-proteins [10]. The central portion of the i3 loop can be deleted without impairing coupling to G-proteins, indicating that only the N- and C-terminal regions of the i3 loop (Ni3 and Ci3, adjacent to TM5 and TM6, respectively) are required for function [24–27]. However, it is clear from those studies that other epitopes besides the i3 loop are required for directing coupling to specific G-proteins because although the coupling selectivity of these chimeric receptors was reversed by exchanging the i3 loop, these receptors gained only a limited capability to couple new G-proteins [22]. Chimeras between the m1 muscarinic receptor and the β3 adrenergic receptor in which the i3 loop was exchanged retained the ability to couple their normally preferred G-protein(s) [28], and further investigation indicated that multiple epitopes contribute to defining receptor selectivity for G-proteins [29]. The results of the present study indicate that the i2 loop also contributes significantly to defining receptor subtype specificity for distinct G-proteins, but that the i1 loops and C-termini of m2 and m5 are functionally interchangeable.

The receptors with m2 segments reaching from the N-terminus to TM2 (CR1) and TM6 (CR4) had enhanced activity relative to m5. These effects were synergistic because CR6,

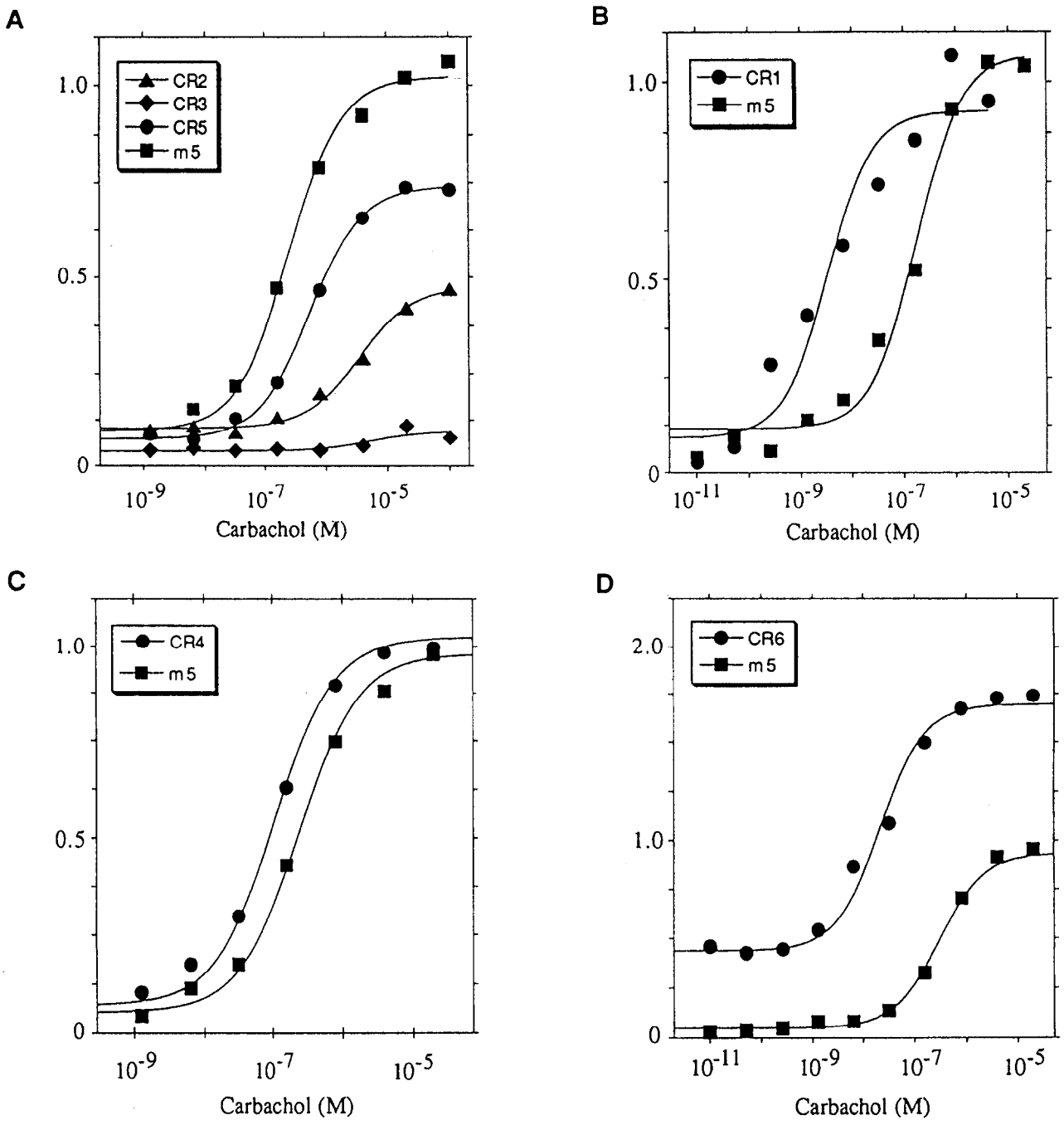

FIG. 3. Activity of m2/m5 chimers in R-SAT. Assays were performed as described in Fig. 1 and in Materials and Methods. m5 is shown for comparison. All results were normalized to the response of wild-type m5 with a value of 1.0 representing the maximal response of m5. (A) Chimeras with impaired or no activity in R-SAT compared to m5. (B) CR1 had markedly greater potency than m5 in R-SAT. (C) CR4 had slightly greater potency than m5 in R-SAT. (D) CR6 was hyperactive, had significantly greater potency, and had elevated basal activity in R-SAT compared with m5.

which contained sequence elements derived from both CR1 and CR4, was constitutively activated. It was surprising that exchanging any m2 segments for m5 would cause a gain of function considering that wild-type m2 had no activity in R-SAT. Most current models of helix packing for G-protein-coupled receptors place TM1 adjacent to TM7 [30]. Therefore, it is possible that substituting m2 sequences for TM1, TM6, TM7, and the extracellular loop connecting TM6 and TM7 may have distorted the helical arrangement in such a manner as to mimic or facilitate transition into the active (capable of interacting with G-proteins) conformation of the receptor.

Using R-SAT, we have screened receptors derived from six separate libraries of m5 muscarinic receptors, each containing random mutations in discrete regions thought to be involved in ligand binding and G-protein coupling. The most highly activated receptors isolated to date contain mutations in TM6

TABLE 1. Responses of m2/m5 chimers in the R-SAT assay

	$EC_{50,Cch}$ (nM)	Maximum response (%)	Basal response (%)	$IC_{50,Atr}$ (nM)
m5	231 ± 59	100		
m2		NR		
CR1	24 ± 17	79 ± 17		
CR2	3340 ± 590	40 ± 3		
CR3		NR		
CR4	134 ± 92	102 ± 3		
CR5	581 ± 52	61 ± 10		
CR6	15 ± 4	149 ± 37	38 ± 4	1.7 ± 0.1

Maximum responses were normalized to the maximal response of wild-type m5, which was 0.4 absorbance units at 405 nm (see Fig. 1). Basal response refers to the response in the absence of ligand. Basal response of wild-type m5 was not significantly different from the response of m5 in the presence of 1 μM atropine and was assigned a value of zero. Maximum response for CR6 was calculated using the response in the presence of 1 μM atropine as the baseline. The $EC_{50,Cch}$ is defined as the concentration of carbachol which gives 50% of the maximum response. The $IC_{50,Atr}$ is a measure of the ability of atropine to suppress basal response. Values are means ± SEM and represent three separate experiments done in duplicate. NR = no response.

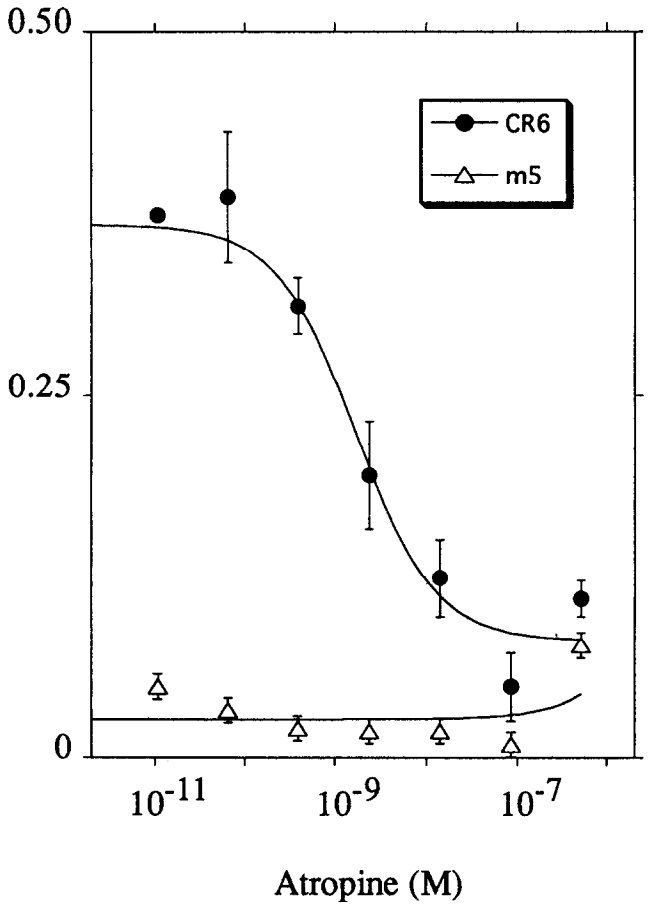

FIG. 4. Suppression of the constitutive activity of CR6 by atropine. R-SAT was performed exactly as described above except that atropine was substituted for carbachol. Values are means ± SEM of three independent experiments. All results were normalized to the response of wild-type m5 with a value of 0.50 representing the half-maximal response of m5.

near the extracellular loop connecting TM6 and TM7 (Spalding *et al.* [19], and unpublished observations). Results from a random mutagenesis experiment on the Ci3 domain [10] suggested that Ci3 forms an α-helical extension from TM6. The closely related adrenergic receptor is activated by mutation of a residue on the extreme intracellular end of this predicted helix [31]. Although mutation of the analogous residue in the m5 muscarinic receptor (alanine 441) does not produce an activated receptor, this residue plays a major role in defining G-protein affinity [10]. Since both muscarinic and adrenergic receptors are activated by mutations at either end of TM6, it is attractive to postulate that receptor activation is caused by a disruption in the packing of TM6 with the other transmembrane domains. This, in turn, leads to speculation that a shift in TM6 may be a vital step in the activation of monoamine receptors. We suspect that a global effect on helix packing is responsible for the observed constitutive activity of CR6, since CR4 was not constitutively activated. More refined structural models of G-protein-coupled receptors will be invaluable for interpreting these results.

This work was supported, in part, by Vermont EPSCOR and PHS R01 GM52737 from N.I.G.M.S. E.S.B. was supported by NRSA Fellowship No. F32NS09436.

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