# Deletion Analysis of the Mouse m1 Muscarinic Acetylcholine Receptor: Effects on Phosphoinositide Metabolism and Down-Regulation†

Robert A. Shapiro and Neil M. Nathanson*

*Department of Pharmacology, SJ-30, University of Washington, Seattle, Washington 98195*

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ABSTRACT: Deletions have been constructed in the putative third cytoplasmic loop of the mouse m1 muscarinic acetylcholine receptor (mAChR) gene, and the effects of these mutations on mAChR coupling to phosphoinositide metabolism and agonist-induced down-regulation have been examined following expression in Y1 adrenal carcinoma cells. Deletion of up to 123 of the 156 amino acids in this loop has no effect on antagonist or agonist binding, or on coupling to stimulation of phosphoinositide metabolism. These results suggest that the membrane proximal portions of this loop are involved in determining the specificity of functional coupling of the receptor. Deletion of 75% of the loop has no effect on short-term agonist-induced internalization but does cause a significant decrease in the magnitude of agonist-induced down-regulation of receptor number. Thus, this portion of the receptor may be involved in mediating the response to long-term agonist exposure.

Muscarinic acetylcholine receptors (mAChRs)[1] are the predominant cholinergic receptors in the central nervous system and play a major role in regulating the functions of the target organs of the parasympathetic nervous system. Activation of mAChRs by acetylcholine can result in a wide variety of cellular responses including changes in intracellular levels of cAMP and cGMP, activation of phospholipase C, and the opening or closing of ion channels (Nathanson, 1987).

Genomic and cDNA clones encoding mAChRs have been isolated from pig (Kubo et al., 1986a, b; Peralta et al., 1987b), human (Peralta et al., 1987a; Bonner et al., 1988), rat (Bonner et al., 1987, 1988; Braun et al., 1987), and mouse (Shapiro et al., 1988). At least five receptor subtypes have been identified and found to be the products of distinct genes. [Several types of nomenclature have been used to distinguish the various receptor subtypes; the nomenclature of Bonner et al. (1987, 1988) will be used here to identify mAChR subtypes.] The results to date suggest that the distinct mAChR subtypes regulate different signal transduction systems. Thus, expression of the m1 and m2 receptors in oocytes leads to the regulation of different ion channels (Fukuda et al., 1987). The murine m1 receptor stimulates phosphoinositide turnover, and the porcine m2 receptor inhibits adenylate cyclase formation when they are expressed in either murine L cells or murine Y1 adrenal carcinoma cells (Shapiro et al., 1988). The neuroblastoma–glioma hybrid cell line NG108-15, in which the mAChR inhibits adenylate cyclase but does not stimulate phosphoinositide breakdown, expresses only a single receptor subtype, m4 (Harden et al., 1986; Peralta et al., 1987a). Fukuda et al. (1988) expressed mAChR subtypes 1–4 in NG108–15 cells and found that the m1 and m3 receptors, but not the m2 and m4 receptors, stimulated phosphoinositide breakdown and suppressed the M-current potassium channel. The functional specificity of the mAChR subtypes may not be absolute, however, as there appears to be some dependence on cell type and receptor levels (Ashkenazi et al., 1988, 1989; Stein et al., 1988).

Muscarinic receptors belong to a family of receptors which are involved in the activation of various signal transduction pathways through their interaction with guanine nucleotide binding proteins (G-proteins). This family includes at least five subtypes of mAChR (Peralta et al., 1987a; Bonner et al., 1987): the serotonin receptors (Julius et al., 1988), the $\alpha$- and $\beta$-adrenergic receptors (Dixon et al., 1986; Yarden et al., 1986; Kobilika et al., 1987), the opsin pigments (Nathans et al., 1986), the dopamine receptors (Bunzow et al., 1988), the substance K receptor (Masu et al., 1987), and a yeast pheromone receptor (Burkholder & Hartwell, 1985). These receptors all share a predicted seven transmembrane domain structure, as revealed by hydrophobicity analysis of deduced amino acid sequences. The amino acid sequences of the vertebrate receptors are highly conserved, especially within certain transmembrane regions. The region of least homology occurs in a proposed cytoplasmic loop located between the fifth and sixth transmembrane regions. This has led to the suggestion that this loop may be involved in the interaction of the receptors with specific G-proteins (Dohlman et al., 1987). In support of this hypothesis, small deletion mutations within this loop in the $\beta$-adrenergic receptor result in the complete loss of receptor–G-protein coupling (Dixon et al., 1987; Strader et al., 1987). The most convincing evidence is the demonstration that switching a portion of the $\alpha$- and $\beta$-adrenergic receptors containing this region causes a shift in the physiological response of the chimeric receptor (Kobilka et al., 1988). Although small deletion mutations in the other cytoplasmic loops do lower the magnitude of the agonist-dependent response, the possibility that these changes may be due to alterations in the overall structure of the receptor rather than in the postulated G-protein binding site cannot be ruled out. Similarly, switching the entire third cytoplasmic loop between the m1 mAChR and the m2 mAChR causes a shift in the

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* To whom correspondence should be addressed.

[1] Abbreviations: mAChR, muscarinic acetylcholine receptor; QNB, quinuclidinyl benzilate; Ins-P, inositol phosphate(s); G-protein, guanine nucleotide binding protein; NMS, *N*-methylscopolamine; kb, kilobase(s).

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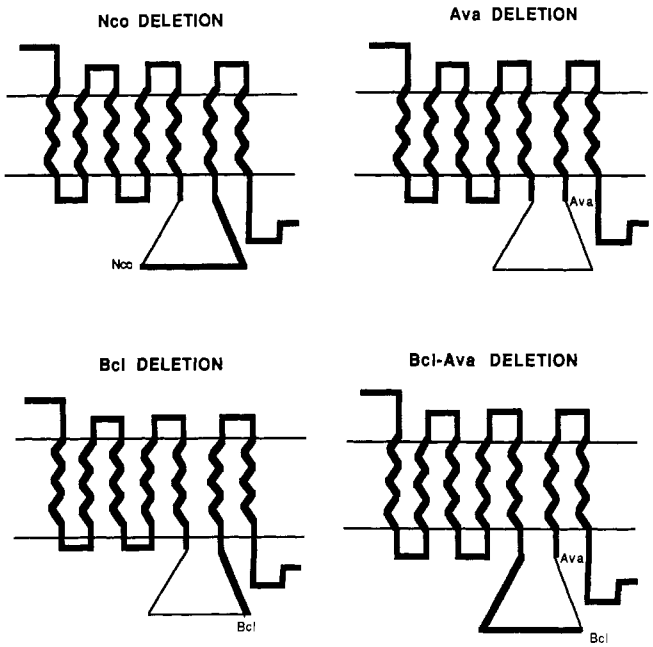


FIGURE 1: Structure of mAChR deletion mutations. The thin line represents the region of the receptor that was deleted. Restriction enzyme names refer to the restriction site which was used in the construction of the deletion mutation (see Materials and Methods).

ability of the hybrid receptors to couple to specific ion channels in oocytes (Kubo et al., 1988). Thus, the amino acid residues contained within the third cytoplasmic loop appear to be sufficient to determine the specificity of mAChR coupling to their respective G-proteins.

In order to more precisely define the portion of the third cytoplasmic loop responsible for the specific coupling of the m1 mAChR, we have constructed deletion mutations in the third cytoplasmic loop of the mouse m1 receptor gene, expressed the altered receptors in Y1 adrenal carcinoma cells, and characterized the effect of these mutations on the ability of the expressed receptor to stimulate phosphoinositide metabolism and to undergo agonist-dependent internalization and down-regulation.

## Materials and Methods

*Construction of Deletion Mutations.* A 1.8-kb *Kpn–Bam*HI fragment containing the entire coding region of the mouse m1 mAChR was subcloned into pGEM3 (Shapiro et al., 1988). Four mAChR deletion mutations (*Ava*Δ, *Nco*Δ, *Bcl*Δ, and *Bcl–Ava*Δ) were constructed as described below (Figure 1). *Ava*Δ: The 1.8-kb m1/pGEM was digested with *Ava*I, filled in with Klenow and a *Bam*HI linker attached, cut with *Bam*HI, and religated. The resulting subclone (m1 5′-*Ava/Bam*) contains the amino-terminal end of the m1 receptor up to amino acid 220. The m1 5′-*Ava/Bam* fragment was then linearized with *Bam*HI and ligated to a 550 bp *Ava*I/*Sma*I fragment with *Bam*HI linkers attached. The resulting construct deletes amino acids 221–343 and replaces them with three amino acids: Arg-Ile-Arg. *Nco*Δ: m1 5′-*Ava/Bam* was linearized with *Bam*HI and ligated to a 3′ 800 bp *Nco*I/*Sal*I fragment (which was filled in with Klenow and had *Bam*HI linkers attached). The resulting construct deletes amino acids 221–284 and replaces them with the amino acids Arg-Ile-Arg. *Bcl*Δ: m1 5′-*Ava/Bam* was linearized with *Bam*HI and ligated to a 3′-*Bcl*I/*Bam*HI fragment (750 bp). The resulting construct deletes amino acids 221–301 and replaces them with the amino acid Arg. *Bcl/Ava*Δ: The 1.8-kb/pGEM was digested with *Bcl*I and *Bam*HI and the vector sequence and 5′ m1 sequence isolated. This fragment was then ligated to the same 3′-*Ava*I/*Sma*I m1 fragment used in the construction of the *Ava*Δ. The resulting construct deletes amino acids 303–343 and replaces them with three amino acids: Arg-Ile-Arg.

*Transfection and Cell Culture.* All deletion constructs were then digested with the appropriate restriction enzymes to remove the coding region from pGEM, filled in with Klenow, and purified on agarose gels. The blunt-ended fragments were then ligated into the filled-in *Bam*HI site of the expression vector ZEM 228 (E. Mulvihill, Zymogenetics). Mouse Y1 adrenal carcinoma cells were then transfected and stable transfectants isolated in the presence of Geneticin (G418) as described (Shapiro et al., 1988).

*Binding Assays in Crude Homogenates.* The binding of [$^3$H]QNB to mAChR in crude membrane homogenates of Y1 cells was performed as described (Shapiro et al., 1988).

*Binding Assay with Intact Cells.* The binding of [$^3$H]NMS to cell surface mAChR on intact cells was performed as described (Nathanson, 1983; Liles et al., 1986) except that incubations with [$^3$H]NMS were done at 4 °C for 4 h to prevent recycling of the receptor to the cell surface.

*Phosphoinositide Hydrolysis.* Cells were incubated overnight in media containing 1 μCi/mL [$^3$H]*myo*–inositol and 120 μM zinc sulfate. The stimulation of phosphoinositide hydrolysis induced by muscarinic agonists was measured as described (Shapiro et al., 1988).

## Results

Deletion mutations in the third cytoplasmic loop of the mouse m1 mAChR gene were constructed from a 1.8-kb genomic DNA clone (Shapiro et al., 1988) as described under Materials and Methods (see Figure 1). All constructs, with the exception of *Bcl–Ava*Δ, contain 11 amino acids common to the wild-type receptor on the amino-terminal end of the third cytoplasmic loop. Deletions then extend toward the carboxy terminal. *Nco*Δ, *Bcl*Δ, and *Ava*Δ remove 64, 82, and 123, respectively, out of the 156 amino acids in the loop. *Bcl–Ava*Δ has the same 21 amino acids on the carboxy-terminal side of the loop as *Ava*Δ but retains all the amino acids deleted in the *Bcl*Δ construct. The entire coding region of each construct was then subcloned into the zinc–inducible expression vector ZEM228 and transformed into mouse Y1 adrenal carcinoma cells (Shapiro et al., 1988) as described under Materials and Methods.

*Ligand Binding.* Stable cell lines arising from transfection with each construct were tested for the ability to bind the mAChR-specific antagonist [$^3$H]QNB. Cells were induced with 120 μM zinc for 24 h, crude membranes were prepared, and the binding of [$^3$H]QNB was determined. Cells expressing all constructs were able to bind 500–1000 fmol of [$^3$H]-QNB/mg of membrane protein. Figure 2A shows a saturation binding curve for the largest deletion mutation, *Ava*Δ. Scatchard analysis of the binding data indicates a $K_D$ of 79 pM, as compared to the $K_D$ of 78 pM previously determined for the wild-type m1 receptor in Y1 cells (Shapiro et al., 1988). Cells were then tested for the ability to bind the agonist carbachol. Crude membranes from zinc-induced cells were incubated with [$^3$H]QNB and increasing amounts of carbachol. The results are shown in Figure 2B. No significant difference in carbachol binding between the wild-type receptor and the *Nco*Δ or *Ava*Δ receptor is observed. Because the wild-type m1 receptor does not exhibit guanine nucleotide dependent regulation of agonist binding when expressed in Y1 cells (Shapiro et al., 1988), it was not possible to test if the deletions had an effect on the sensitivity of agonist binding

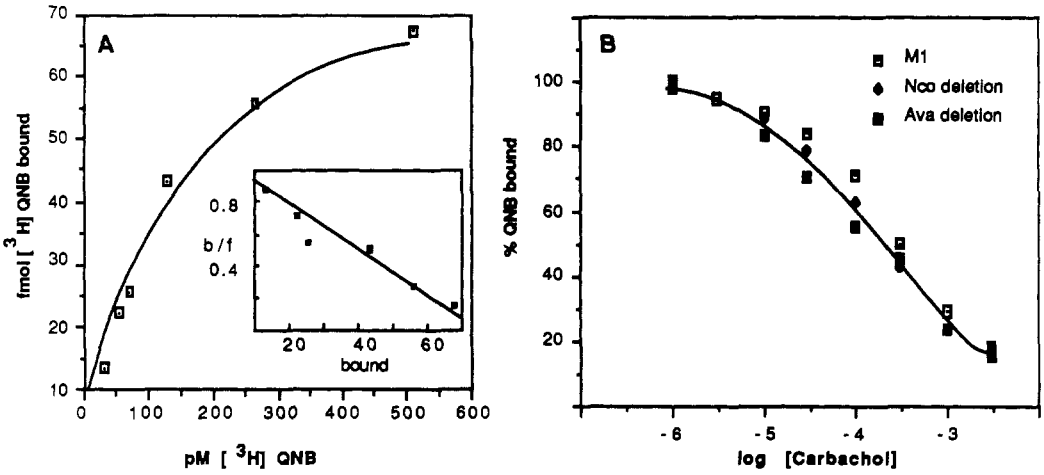


FIGURE 2: (A) [$^3$H]QNB binding to m1 *Ava*Δ in membrane homogenates of transfected Y1 cells. Duplicate assay tubes contained 40 μg of crude membrane protein and increasing concentrations of [$^3$H]QNB. Specific binding was determined by subtracting the amount bound in the presence of 1 μM atropine. Scatchard analysis (inset) of the binding data indicates a $K_D$ of 79 pM. (B) Carbachol competition of [$^3$H]QNB binding. Crude membranes (25 μg of protein) from zinc-induced (125 μM) Y1 cells expressing the m1 wild-type mAChR, m1 *Nco*Δ, and m1 *Ava*Δ were incubated with [$^3$H]QNB (350–410 pM) and increasing amounts of carbachol. The amount of [$^3$H]QNB was determined as described under Materials and Methods. The levels of the m1 receptor, *Nco*Δ receptor, and *Ava*Δ receptor in the transfected Y1 cells were 1000, 950, and 925 fmol/mg of membrane protein, respectively.

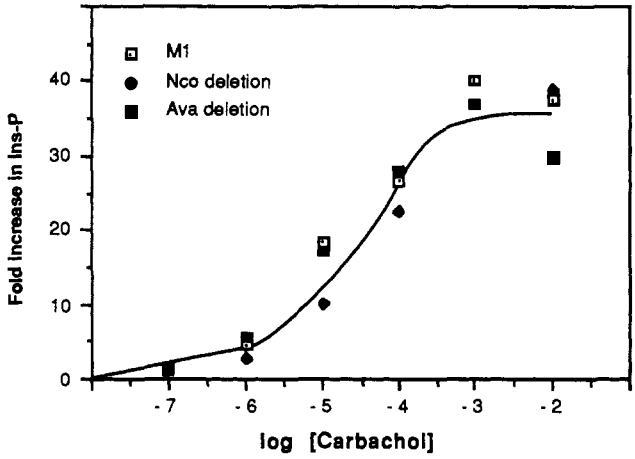


FIGURE 3: Receptor-mediated stimulation of phosphoinositide metabolism. Triplicate 35-mm plates of Y1 cells transfected with either m1 mAChR or the indicated deletion mutations were incubated with increasing concentrations of carbachol for 15 min at 37 °C, and the production of total [$^3$H]inositol phosphates was determined as described. The results are presented as the increase in total [$^3$H]inositol phosphates formed above the basal level measured in the absence of carbachol, which was 70 cpm. The levels of m1 receptor, *Nco*Δ receptor, and *Ava*Δ receptor were 1000, 900, and 950 fmol/mg of membrane protein, respectively, as measured by [$^3$H]QNB binding.

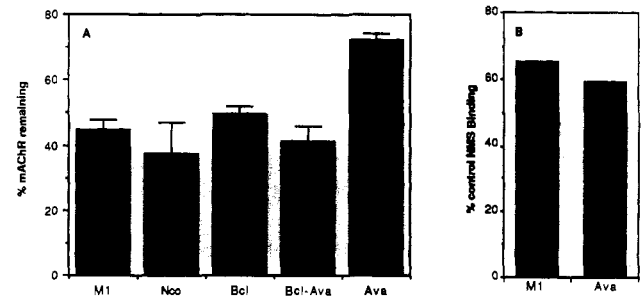


FIGURE 4: (A) Long-term agonist-mediated down-regulation of receptor number. Triplicate 60-mm plates of mAChR-transfected Y1 cells were induced with 125 μM zinc for 24 h. Zinc-containing medium was then removed, replaced with fresh medium in the absence or presence of 1 mM carbachol, and incubated for 12 h. Membranes were perpared, and receptor density was determined by [$^3$H]QNB binding. The data represent the average of two experiments. (B) Short-term agonist-induced receptor internalization. Triplicate 35-mm plates of Y1 cells transfected with either m1mAChR or *Ava*Δ were induced with 100 μM zinc for 24 h. Plates were then washed and exposed to 1 mM carbachol for 20 min. [$^3$H]NMS binding to cell surface mAChR on intact cells was determined as described under Materials and Methods. Data are expressed as percent of control values (no added carbachol). The data represent the average of triplicate values which varied less than 10%.

to guanine nucleotides. However, the data in Figure 2 do demonstrate that most of the third cytoplasmic loop is not required for ligand binding to the m1 mAChR.

*Phosphoinositide Turnover.* *Ava*Δ and *Nco*Δ were then tested for the ability to stimulate phosphoinositide turnover in response to the agonist carbachol. Figure 3 shows the results of increasing concentrations of carbachol on the production of total inositol phosphates. Cells containing either deletion were able to stimulate the production of total inositol phosphates to the same levels and with identical carbachol dose–response curves as the wild-type receptor. Thus, amino acids 221–343 are not required for coupling of the m1 mAChR to increases in phosphoinositide turnover.

*Agonist-Induced Down-Regulation of mAChR Number.* In order to test the ability of the m1 deletion mutants to evoke another agonist-induced response, *Ava*Δ and *Nco*Δ were tested for the susceptibility to undergo long-term agonist-mediated decreases in receptor number. Receptor expression was induced by the addition of zinc as described. The zinc-containing medium was then removed and replaced with either fresh medium or medium containing 1 mM carbachol. After 12 h, crude membranes were prepared, and the total number of receptors was measured by [$^3$H]QNB binding is described. The level of receptor in cells expressing the *Nco*Δ receptor was reduced to the same extent as the wild-type receptor (40–45% of control levels). The m1 *Ava*Δ was reduced to only 75% of control [$^3$H]QNB binding sites. In order to attempt to localize the region responsible for the observed decrease in the extent of agonist-dependent down-regulation, two other deletions were constructed which restored either the N-terminal (*Bcl–Ava*Δ) or the C-terminal (*Bcl*Δ) half of the missing portion of the cytoplasmic loop (see Materials and Methods and Figure 1). Agonist-induced down-regulation of the wild type and the four deletion mutations was determined as described above. The results are summarized in Figure 4A. The receptor levels in

the wild-type m1 and in the *Nco*Δ, *Bcl*Δ, and *Bcl-Ava*Δ mutants were all reduced to 40–50% of the receptor levels in untreated cells, while *Ava*Δ was reduced to 75% of control values.

The m1 receptor in Y1 cells is rapidly internalized by the addition of the agonist carbachol (Scherer et al., 1988). To assess the possibility that the impaired ability of *Ava*Δ to undergo long-term agonist-mediated down-regulation was due to a defect in the ability of the receptors to be internalized (and thus not be subsequently degraded), short-term agonist-dependent internalization of the receptor was measured by using the labeled antagonist [$^3$H]NMS. [$^3$H]NMS is membrane-impermeable and thus will only bind to cell surface receptors when incubated with intact cells (Galper et al., 1982; Feigenbaum & El-Fakahany, 1985). Cells were induced with zinc as described and treated with 1 mM carbachol for 20 min, and cell surface [$^3$H]NMS binding sites were determined. The results are shown in Figure 4B. No difference in agonist-mediated internalization is observed between the wild-type receptor and the *Ava*Δ.

## Discussion

Muscarinic receptors belong to a large family of membrane-bound receptors that are coupled to intracellular second-messenger systems by their interaction with specific members of a large family of G-proteins. All members of this receptor gene family exhibit a remarkable level of amino acid homology, especially within the transmembrane regions. The regions of least homology occur in the proposed third cytoplasmic loop, leading to the proposition by Dohlman et al. (1987) that the third cytoplasmic loop may provide the specificity for the receptor/G-protein interaction. Results from several laboratories support this hypothesis. Small deletion mutations within this loop in the β-adrenergic receptor result in complete loss of receptor/G-protein coupling (Dixon et al., 1987; Strader et al., 1987), while switching a portion of the α- and β-adrenergic receptors containing this region causes a shift in the physiological response of the chimeric receptor (Kobilka et al., 1988). Using the adrenergic receptor system as a model, one would predict that the membrane proximal portions of the third cytoplasmic loop are major determinants of the receptor/G-protein interaction. This model is further supported by the results of Franke et al. (1988) which showed that a single amino acid substitution in this region of rhodopsin prevents its activation of transducin. The results of Kubo et al. (1988) using the porcine m1 and m2 mAChRs are also consistent with this model. The specificity of hybrid mAChRs for coupling to different ion channels was determined by the source of this cytoplasmic loop. Thus, chimeric m1–m2 mAChRs containing only the third cytoplasmic loop of the m1 mAChR were able to elicit to m1-type currents after expression in oocytes. These results provide strong evidence that the third cytoplasmic loop is sufficient to determine the specificity of mAChR coupling to physiological responses.

The results presented in this report demonstrate that 75% of this loop is not necessary for the coupling of the m1 mAChR to increases in phosphoinositide turnover. Furthermore, in light of the results of Kubo et al. (1988), demonstrating the role of the third cytoplasmic loop in the determination of functional specificity, these results predict that the determinants of m1 mAChR/G-protein coupling are contained within amino acids 209–220 and 344–365.

Previous work has demonstrated that exposure of cells to muscarinic agonists causes a rapid (with a time scale of minutes) internalization of the mAChR. Internalization can be detected as a loss of receptors which are accessible on intact cells for the binding of membrane-impermeable ligands such as NMS. The continued presence of agonist causes a subsequent decrease in the total number of cellular receptors due to an increased rate of receptor degradation (Klein et al., 1979; Galper et al., 1982; Feigenbaum & El-Fakahany, 1985; Liles et al., 1986). *Ava*Δ did not exhibit a decrease in agonist-induced internalization, indicating that the decrease in agonist-induced down-regulation was not due to impaired receptor internalization. Perhaps the removal of the major portion of the third cytoplasmic loop stabilizes the receptor against degradation after it is internalized. There does not appear to be a unique site required for this stabilization as both *Bcl*Δ and *Bcl–Ava*Δ exhibit the same extent of down-regulation as the native receptor. It is possible that the smaller loop in *Ava*Δ is less accessible to intracellular proteases than receptors with larger cytoplasmic loops. Alternatively, a portion of the cytoplasmic loop may be required for targeting internalized receptors to lysosomes for degradation.

In conclusion, we have demonstrated in this paper that the third cytoplasmic loop of the mAChR is important in long-term agonist-mediated down-regulation of receptor number. We also demonstrate that the major portion of the third cytoplasmic loop of the m1 mAChR is not required for coupling of receptor to phosphoinositide turnover. Because of the strong evidence that the third cytoplasmic loop of mAChRs is sufficient to determine the specificity of G-protein coupling of receptor to intracellular physiological responses, it is most likely that the determinants of G-protein coupling to receptor reside in the remaining membrane proximal amino acids.

**Registry No.** Carbachol, 51-83-2.

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