Differential Role of the Carboxyl-terminal Tyrosine in Down-regulation and Sequestration of the m2 Muscarinic Acetylcholine Receptor*

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The muscarinic acetylcholine receptors (mACHRs) are members of a superfamily of receptors which contain seven transmembrane domains and elicit their physiological effects through interactions with guanine nucleotide binding proteins (G proteins). There are five mammalian mACHR subtypes which preferentially couple to either the inhibition of adenylate cyclase activity (m2 and m4 subtypes) or to the stimulation of phospholipase C activity (m1, m3, and m5 subtypes) (Kubo et al., 1986a, 1986b; Peralta et al., 1987a, 1987b; Bonner et al., 1987, 1988). The number of mACHR expressed in cells or tissues can be altered by continued exposure of agonist leading to a decrease in responsiveness to further stimulation by agonist (Nathanson, 1989). Short term agonist exposure (seconds to minutes) causes sequestration of receptors to a state where it is inaccessible to binding of lipophobic ligands (Galper et al., 1982), perhaps due to internalization of receptors from the cell surface into an intracellular light vesicle fraction (Harden et al., 1985). Upon agonist removal, sequestered receptors are quickly (minutes) recycled to their original state (Maloteaux et al., 1983; Feigenbaum and El-Fakahany, 1985). In contrast, prolonged agonist exposure (hours) results in a decrease in total mACHR number, and recovery requires several hours and de novo protein synthesis (Klein et al., 1979; Taylor et al., 1979; Hunter and Nathanson, 1984). This “down-regulation” of mACHR results from an increase in degradation of receptor protein (Klein et al., 1979; Galper and Smith et al., 1980).

The mechanisms involved in the sequestration and down-regulation of the mACHR have not been clearly defined. Likewise, few specific receptor domains involved in the regulation of mACHR number have been identified (Morro et al., 1993). Tyrosine residues located in the cytoplasmic tails of many membrane receptors are necessary for agonist-induced internalization of these receptors via clathrin coated pits (Mostor et al., 1986; Davis et al., 1987; Iaccopetta et al., 1988; Chen et al., 1990). In addition, either of the 2 tyrosine residues located in the cytoplasmic tail of the β2-adrenergic (β2AR) is required for the down-regulation but not the sequestration of receptors in response to agonist (Valiquette et al., 1990). In this report, we use site-directed mutagenesis to test the role of the single carboxyl-tail tyrosine residue in the mammalian m2 receptor in receptor regulation and function. We demonstrate that substitution of Tyr-459 with Phe, Trp, Ala, or Ile does not affect agonist or antagonist binding, functional coupling to the inhibition of CAMP accumulation or agonist-induced sequestration of the m2 receptor. However, the sensitivity as well as the rate and extent of agonist-induced down-regulation was attenuated in the Ala, Phe, and Trp mutant m2 receptors. The Tyr to Ile replacement decreased the sensitivity of m2 to agonist-induced down-regulation slightly, although the maximal down-regulation of the Ile mutant over time was not significantly different from wild-type m2. These results are the first to identify a site in a mACHR involved in the down-regulation of receptor in response to agonist.

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The abbreviations used are: mACHR, muscarinic acetylcholine receptor; G protein, guanine nucleotide-binding protein; β2AR, β2-adrenergic receptor; PCR, polymerase chain reaction; QNB, quinuclidinyl benzilate; NMS, N-methylscopolamine; CRE, CAMP response element; PBS, phosphate-buffered saline.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Construction of Expression Vectors—Site-directed mutagenesis of Tyr-459 of the porcine m2 mACHR (clone Mc7) (Peralta et al., 1987b) (a gift from D. Capon, Genentech Inc.) was performed using the polymerase chain reaction (PCR). Oligonucleotides were synthesized by the University of Washington Molecular Pharmacology Facility. Mutagenic oligonucleotides were complimentary to nucleotides 1357-1403 and contained single or double base changes to replace Tyr-459 with either an Ala (Y459A); Trp (Y459W); Phe (Y459F), or Ile (Y459I). The 5′ oligonucleotides corresponded to nucleotides –31 to –14. PCR reactions were performed with Taq DNA polymerase (Promega, Madison, WI) utilizing reaction conditions described by the manufacturer with denaturation at 95 °C for 5 min, annealing at 60 °C
for 2 min, and elongation at 70 °C for 3.5 min for a total of 30 cycles. Both the 3' and 5' oligonucleotides contained EcoRI sites adjacent to receptor sequence. The PCR products were subcloned into the EcoRI site of pGEM-3Z (Promega), and Applied Biosystems, Inc. (Foster City, CA) Taq Dye Primer Cycle Sequencing analysis verified that the single amino acid changes at Tyr-459 were the only mutations in the carboxyterminal 120 residues of the mutants. The constructs were digested with BsiI and NdeI, and the fragments containing nucleotides 1352–1405 of the mutated m2 mAChR were gel-isolated and ligated with a BsuNI/NdeI fragment containing nucleotides –31 to 1351 isolated from the wild-type m2 mAChR subcloned in pGEM-3Z. The resulting mutant mAChR were digested with EcoRI and ligated into the expression vector pCD-F (Bonner et al., 1988) (a gift from T. Bonner, NIH, Bethesda, MD).

Cell Culture and Transfection—The human choriocarcinoma cell line JEG-3 (American Tissue Culture Collection, Rockville, MD) was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies, Inc.), penicillin G (100 units/ml), and streptomycin sulfate (0.1 mg/ml) (Apothecon, Princeton, NJ) in a 10% CO2 environment at 37 °C. Cells plated on 150-mm tissue culture dishes were transiently transfected with 35–60 µg of wild-type or Tyr-459 mutant m2 receptors via the calcium phosphate precipitation method (Santer et al., 1986).

Ligand Binding—The binding of the muscarinic antagonist [3H]quinuclidinyl benzilate ([3H]QNB, Amersham Corp., 47 Ci/mmol) to mAChR in crude membrane homogenates and carbachol ([3H]QNB) competition assays were performed as described by Halvorsen and Nathanson (1981). Nonspecific binding was determined as the binding which remained in the presence of 1 µM atropine. Carbachol and atropine were obtained from Sigma. In the carbachol ([3H]QNB) competition assays, the concentration of [3H]QNB was ~500 pM. K0 values of [3H]QNB and EC50 values of carbachol were determined by linear regression analysis using the program GraphPad InPlot version 3.0 (GraphPAD Software, San Diego, CA).

CAMP Inhibition Assays—JEG-3 cell transfection and culture as well as assays of luciferase and β-galactosidase activities were performed as described by Migeon and Nathanson (1994). Transfection mixes contained 75 ng/well Gαi3 (Jones and Reed, 1989) in the expression vector pCD-F, 15 ng/well CRE-luciferase plasmid which contains a luciferase reporter gene driven by a CAMP-regulated 168-base pair promoter from the α-glycoprotein hormone gene (Mellon et al., 1989), 40 ng/well Rous sarcoma virus β-galactosidase plasmid (Edlund et al., 1986), 20 ng/well mAChR expression vector plasmid, and 100 ng/100 µl pCD-F carrier.

Sequestration Assays—The binding of N-[3H]methylscopolamine ([3H]NMS, Amersham Corp., 47 Ci/mmol) to intact cells was previously described (Nathanson et al., 1992). Briefly, 24 h after transfection, the cells from one 150-mm plate were replated onto five 6-well culture dishes and allowed to attach for 24 h. The cells were incubated with various concentrations of carbachol for 5–60 min (6 wells per treatment) while 12 wells (control) received no drug treatment. All cells were washed on ice three times with ice-cold phosphate-buffered saline (PBS, 20 mM NaH2PO4, 150 mM NaCl, pH 7.4) and incubated on ice with 1 nM [3H]NMS in 2 ml of ice-cold PBS. Four control wells received atropine (1 µM) to measure nonspecific binding. After a 4-h incubation, the cells were washed on ice three times with ice-cold PBS, resuspended with 0.5 µl of 1% Triton X-100, scraped, and transferred to scintillation vials, which received 3.5 ml of scintillation fluid prior to scintillation counting.

Down-regulation Assays—Transfection and plating were carried out as above. The cells were incubated with various concentrations of carbachol for 0.5–8 h (6 wells per treatment) while 12 wells (control) received no treatment. The cells were washed three times with warm assay buffer (116 mM NaCl, 1.8 mM CaCl2, 5.4 mM KC1, 0.81 mM MgSO4, 1.0 mM NaH2PO4, 25 mM glucose, and 25 mM Hepes, pH 7.4), 2 ml of warm assay buffer were added to each well, and the plates were incubated at 37 °C for 15 min. Each well then received 0.45 µM [3H]QNB while four control wells also received atropine (1 µM). After incubation at 37 °C for 1.5 h, the cells were washed with ice-cold PBS. Two ml of ice-cold PBS were then added to each well, and the plates were placed on ice until being scraped and filtered over GF/C filters (Whatman). The filters were washed three times with 5 ml of ice-cold PBS and placed in 3.5 ml of scintillation fluid. The radioactivity was determined by scintillation counting.

RESULTS

Expression and Ligand Binding—Four mutant m2 receptors were constructed by PCR-based mutagenesis, and sequence analysis confirmed that the mutant m2 receptors contained only a single amino acid replacement at Tyr-459 (Tyr-459 substituted by Phe, Trp, Ala, or Ile). All four Tyr-459 mutant m2 mAChR were transiently expressed in JEG-3 cells at similar levels as wild-type m2 mAChR (data not shown). The levels of receptor expression ranged from 450 to 8000 fmol/mg of membrane protein in different experiments. No difference in the percentage of sequestration or down-regulation was observed with either wild-type m2 or Tyr-459 mutants over the same range of expression levels (data not shown).

[3H]QNB saturation binding experiments were performed in order to determine the dissociation constants (Kd) of the mutant receptors to carbachol, which were not significantly different from wild-type m2 (Table I) and were similar to Kd values of other mAChR (Peralta et al., 1987b; Liles et al., 1986).

Competition binding experiments with [3H]QNB and increasing concentrations of carbachol showed no significant differences in EC50 values for carbachol between the mutant and wild-type mAChR. Table I shows that the wild-type and mutant mAChR receptors to inhibit forskolin-stimulated CAMP accumulation was measured by assay of receptor-mediated inhibition of expression of a luciferase receptor gene under the transcriptional control of a CAMP-responsive element (CRE) as previously described (Migeon and Nathanson, 1994). The m2 and Tyr-459 mutant mAChR receptors were co-transfected with Gαi3, α168 CRE-luciferase, a constitutive expression plasmid for β-galactosidase, and pCD-F carrier. β-Galactosidase activity was measured to normalize for slight differences in transfection efficiency. Fig. 1 shows that the wild-type and mutant mAChR mediated inhibition of forskolin-stimulated luciferase expression in response to increasing concentrations of carbachol in a dose-dependent manner with the greatest inhibition occurring at 10−6 M carbachol. The mutant mAChR receptors showed similar levels of inhibition of luciferase expression compared to the wild-type m2, indicating that Tyr-459 is not necessary for agonist-induced inhibition of CAMP accumulation (Fig. 1). Except for the Y459I mutant, higher levels of carbachol (10−5 M) caused a decrease in luciferase expression most likely due to increased CAMP accumulation through coupling of mAChR to Gαs (Migeon and Nathanson, 1994). The Y459I mutant's apparent increased extent of inhibition of CAMP accumulation and apparent lack of attenuation of inhibition of CAMP accumulation at a high concentration of carbachol is under investigation.
Involvement of Tyr-459 in m2 Receptor Down-regulation

FIG. 1. Luciferase activity of wild-type and Tyr-459 mutant m2 mAChR. JEG-3 cells transiently transfected with wild-type (●) or Tyr-459 mutant (■ Y459F, □ Y459F, △ Y459W, O, Y459I) mAChR were incubated with 10^-3 -10^-7 M carbachol and 0.316 µs forskolin for 4 h at 37°C. Luciferase activity of mAChR was assayed as described and is presented as a fold increase in the luciferase activity of control cells receiving no carbachol. All data for wild type and mutant mAChR were presented as a fold increase in luciferase activity of control cells. Y459F and Y459I were normalized by assay of β-galactosidase activity and represent the mean ± S.E. of four to eight experiments and are expressed as a percentage of [3H]NMS binding sites in untreated cells. Only the sequestration of Y459F at 10^-6 M and Y459A at 10^-5 M carbachol are significantly different from wild type (p < 0.05).

mAChR—The effect of carbachol pretreatment on the sequestration of the wild-type and mutant m2 receptors was measured using the membrane-impermeable muscarinic antagonist [3H]NMS (Galper et al., 1982; Harden et al., 1985). Incubation with increasing concentrations of carbachol (10^-3 -10^-7 M) for 15 min caused a dose-dependent decrease in [3H]NMS binding to m2 (Fig. 2). [3H]NMS binding to wild-type receptor was decreased by 21% when preincubated with 1 µM carbachol for 15 min, and was decreased by 41% when incubated for 1 h (Fig. 3). Measurement of the effect on sequestration of m2 after long incubations with carbachol (∼1 h) is complicated since a decrease in total receptor number (down-regulation) begins to occur by this time (see Fig. 5).

The dose-dependent decrease in [3H]NMS binding to Y459F is similar to that of m2 and the decrease in levels of [3H]NMS binding to Y459F at 10^-5 M carbachol is only significantly different from wild-type m2 at 10^-4 M carbachol. The Y459W and Y459F mutants exhibit the same rate of sequestration as the m2 receptor, except that initially (at 5 min of preincubation with carbachol) the mutant m2 receptors are sequestered to a lesser extent than wild-type m2 (Fig. 3A).

Dose-dependent decreases in [3H]NMS binding to Y459I are not significantly different from that of m2 except at 10^-3 M carbachol, where carbachol causes a slight but significantly larger decrease in [3H]NMS binding of Y459I (Fig. 2B). However, excluding the 15-min time point, the sequestration of Y459I and m2 is indistinguishable for up to 1 h of preincubation with 1 µM carbachol (Fig. 3B). Pretreatment with a high concentration of carbachol over time produces similar decreases in [3H]NMS binding between Y459A and m2 (Fig. 3B), although Y459A exhibits more sequestration at 10^-6 M carbachol than wild-type receptor (Fig. 2B). Despite a few differences in either dose- or time-dependent agonist-induced sequestration between wild-type and mutant receptors, we conclude that Tyr-459 does not have a significant role in sequestration since no consistent or overwhelming pattern of inhibition of sequestration was observed.

Effects of Carbachol on the Down-regulation of m2 and Tyr-459 mutant m2 mAChR—The effects of carbachol pretreatment on total receptor number were measured by the binding of the membrane-permeable muscarinic antagonist [3H]QNB (Galper et al., 1982; Harden et al., 1985). Fig. 4 shows that a 2-h pretreatment of m2 with various concentration of carbachol causes a dose-dependent decrease in [3H]QNB binding. When preincubated with 10^-5 M carbachol for 2 h, [3H]QNB binding was decreased from control by 38%, and this down-regulation continues up to 8 h (Fig. 5).

When compared with wild-type receptor, the down-regulation of Y459W m2 and Y459F is markedly attenuated in re-
Involvement of Tyr-459 in m2 Receptor Down-regulation

Fig. 3. Time course of agonist-induced sequestration of wild-type and Tyr-459 mutant m2 mAChR. JEG-3 cells transiently transfected with wild-type (○), Y459F (■), Y459W (△), Y459A (△), or Y459I (□) were pretreated with 1 mM carbachol for the indicated times at 37°C, and the loss of [3H]NMS binding sites was measured as described under Experimental Procedures. Data for (A) wild-type, Y459F, and Y459W and (B) wild-type, Y459A, and Y459I represent the mean ± S.E. of three to seven experiments and are presented as a percentage of [3H]NMS binding sites in untreated cells. Tyr-459 mutant sequestration levels significantly differed (p < 0.05) from wild-type at 5 min (Y459W and Y459F) and 15 min (Y459I).

Fig. 4. Dose response of agonist-induced down-regulation of wild-type and Tyr-459 mutant m2 mAChR. Transiently transfected JEG-3 cells were pretreated with the indicated concentration of carbachol for 2 h at 37°C, and levels of down-regulated receptors were determined by specific binding of [3H]QNB as described under Experimental Procedures. A, data for wild-type (○), Y459F (■), and Y459W (△) and B, wild-type (○), Y459A (△), and Y459I (□) represent the mean ± S.E. of three to five experiments and is expressed as a percentage of [3H]QNB binding sites in untreated cells. The levels of Tyr-459 mutant [3H]QNB binding were significantly different (p < 0.05) from wild type at all points except: 10⁻⁵ M (Y459F, Y459W, and Y459I) and 10⁻⁴ M (Y459A).

Discusson

In this report, we demonstrate the importance of a Tyr-459 residue located in the carboxyl cytoplasmic tail of the m2 mAChR in the down-regulation but not the sequestration of the receptor in response to agonist stimulation. Our results correspond to those of Valiquette et al. (1990) who have reported that two cytoplasmic tail tyrosine residues of the G protein-coupled β2AR are involved in agonist-induced down-regulation but not sequestration of the receptor. Either of the β2AR cytoplasmic tail tyrosines alone is sufficient for down-regulation of the receptor, since only mutation of both tyrosines attenuates down-regulation (Valiquette et al., 1993). In addition, cytoplasmic tail tyrosines are necessary in the regulation of membrane receptors known to undergo endocytosis via clathrin coated pits: the cytoplasmic tail tyrosines of the mannose 6-phosphate (Lobe et al., 1989; Collawn et al., 1990) and the low density lipoprotein receptors (Chen et al., 1990; Bansal and Gierasch, 1991) are important constituents of a β-turn which interacts with clathrin-binding proteins called adaptors (Glickman et al., 1989). In contrast, the mechanisms involved in the sequestration and down-regulation of the members of the G protein-coupled family of receptors are not as well defined, and our data help contribute to the understanding of the cellular processes involved in and the interactions between these two forms of receptor regulation.

Response to 2 h preincubation with increasing concentrations of carbachol as shown in Fig. 4A. The Y459W mutant is significantly less sensitive to down-regulation by carbachol pretreatment than either m2 or Y459F since 10⁻⁵ M carbachol only decreases [3H]QNB binding 2% over control as compared with a 23% and 16% decrease in m2 and Y459F m2 binding, respectively (Fig. 4A).

Fig. 5A shows decreases in [3H]QNB binding in response to 1 mM carbachol incubation over time for the Y459W, Y459F, and wild-type receptors. The down-regulation of Y459W and Y459F are both significantly attenuated at shorter incubations with carbachol (2 h) although at 4 h a similar decrease in [3H]QNB binding in both mutant and wild-type receptors exists. The decreases in [3H]QNB binding between Y459F and m2 remain similar over longer incubations with carbachol (8 h), although the down-regulation of Y459W is significantly attenuated at this time.

The Y459A mutant has a decreased sensitivity to carbachol-induced down-regulation when compared with the wild-type m2 (Fig. 4B). The rate and extent of down-regulation were also attenuated and followed a similar pattern as Y459W (Fig. 5B). Y459I exhibits a decreased sensitivity to agonist-induced down-regulation, although, interestingly, at a high carbachol concentration the rate and extent of maximal down-regulation of Y459I is indistinguishable from that of wild-type m2 (Figs. 4B and 5B).
It is believed that short term agonist exposure results in a rapid sequestration of receptors into an intracellular compartment from which receptors are either recycled back to the cell surface (if agonist is removed) or targeted to lysosomes for degradation (if agonist remains). However, the significant effects of mutations of Tyr-459 on m2 down-regulation but not sequestration indicate that down-regulation of the mAChR is independent of receptor sequestration. This conclusion is supported by the observation that when expressed in JEG-3 cells transiently transfected with wild-type (●), Y459F (■), Y459W (□), Y459A (△), or Y459I (○) were pretreated with 1 μM carbachol for the indicated times at 37 °C, and the loss of [3H]QNB binding sites measured as described under "Experimental Procedures." Data for (A) wild-type, Y459F, and Y459W and (B) wild-type, Y459A, and Y459I represent the mean ± S.E. of three to eight experiments and are presented as a percentage of [3H]QNB binding sites in untreated cells. Tyr-459 mutant down-regulation levels significantly differed (p < 0.05) from wild-type at 0.5 h (Y459I), 1 h (Y459F, Y459A, and Y459I), 2 h (Y459F, Y459W, and Y459A) and 4 h (Y459W and Y459A).

![Graph A](image1.png)

**Graph A** shows the time course of agonist-induced down-regulation of wild-type and Tyr-459 mutant m2 mAChR. JEG-3 cells transiently transfected with wild-type (●), Y459F (■), Y459W (□), Y459A (△), or Y459I (○) were pretreated with 1 μM carbachol for the indicated times at 37 °C, and the loss of [3H]QNB binding sites measured as described under "Experimental Procedures." Data for (A) wild-type, Y459F, and Y459W and (B) wild-type, Y459A, and Y459I represent the mean ± S.E. of three to eight experiments and are presented as a percentage of [3H]QNB binding sites in untreated cells. Tyr-459 mutant down-regulation levels significantly differed (p < 0.05) from wild-type at 0.5 h (Y459I), 1 h (Y459F, Y459A, and Y459I), 2 h (Y459F, Y459W, and Y459A) and 4 h (Y459W and Y459A).

[![Graph B](image2.png)](image2.png)

**Graph B** shows the time course of agonist-induced down-regulation of wild-type and Tyr-459 mutant m2 mAChR. JEG-3 cells transiently transfected with wild-type (●), Y459F (■), Y459W (□), Y459A (△), or Y459I (○) were pretreated with 1 μM carbachol for the indicated times at 37 °C, and the loss of [3H]QNB binding sites measured as described under "Experimental Procedures." Data for (A) wild-type, Y459F, and Y459W and (B) wild-type, Y459A, and Y459I represent the mean ± S.E. of three to eight experiments and are presented as a percentage of [3H]QNB binding sites in untreated cells. Tyr-459 mutant down-regulation levels significantly differed (p < 0.05) from wild-type at 0.5 h (Y459I), 1 h (Y459F, Y459A, and Y459I), 2 h (Y459F, Y459W, and Y459A) and 4 h (Y459W and Y459A).

It is believed that short term agonist exposure results in a rapid sequestration of receptors into an intracellular compartment from which receptors are either recycled back to the cell surface (if agonist is removed) or targeted to lysosomes for degradation (if agonist remains). However, the significant effects of mutations of Tyr-459 on m2 down-regulation but not sequestration indicate that down-regulation of the mAChR is independent of receptor sequestration. This conclusion is supported by the observation that when expressed in JEG-3 cells transiently transfected with wild-type (●), Y459F (■), Y459W (□), Y459A (△), or Y459I (○) were pretreated with 1 μM carbachol for the indicated times at 37 °C, and the loss of [3H]QNB binding sites measured as described under "Experimental Procedures." Data for (A) wild-type, Y459F, and Y459W and (B) wild-type, Y459A, and Y459I represent the mean ± S.E. of three to eight experiments and are presented as a percentage of [3H]QNB binding sites in untreated cells. Tyr-459 mutant down-regulation levels significantly differed (p < 0.05) from wild-type at 0.5 h (Y459I), 1 h (Y459F, Y459A, and Y459I), 2 h (Y459F, Y459W, and Y459A) and 4 h (Y459W and Y459A).

In summary, the cytoplasmic tail tyrosine residue of the m2 mAChR has been shown to occur by an increase in degradation of receptor protein (Klein et al., 1979; Galper and Smith, 1980; Wang et al., 1990). One or more steps in the degradation of m2 appear to require the presence of Tyr-459, while a role for Tyr-459 in ligand binding and coupling can be ruled out (Table I and Fig. 1). Even though we demonstrated that down-regulation of m2 is distinct from sequestration, receptors targeted for down-regulation might enter the cell by a different mechanism than those receptors sequestered by short term agonist exposure, and Tyr-459 might be necessary for internalization of receptors to be down-regulated. If this were the case, however, one would expect an increased proportion of cell surface Y459A receptors compared to wild-type at a 1-h exposure of agonist, which we do not observe (Figs. 3B and 5B). Further studies will be required to discern the mechanism responsible for the involvement of Tyr-459 in the down-regulation process.

The relative importance of Tyr-459 in m2 down-regulation was studied using a series of amino acid substitutions at this residue. Mutating m2 Tyr-459 to Phe or Trp attenuated down-regulation indicating that the aromatic side chain of the Tyr residue is not sufficient for m2 down-regulation. This conclusion is likewise supported by the absence of any difference in the time course of agonist-induced down-regulation between m2 and Y459I (Fig. 5B). The fact that down-regulation of Y459I is similar to the wild-type receptor also suggests that the hydroxyl group of Tyr is not required in agonist-induced down-regulation of m2. This apparent lack of necessary specific structural properties of Tyr-459 in the down-regulation of m2 suggests the importance of Tyr-459 in the context of a larger domain in which subtle changes in amino acid structure or size might perturb the overall structural nature of the motif. Indeed, the sequence of the whole cytoplasmic tail of the m2 receptor is conserved across species, possibly suggesting the existence of a "down-regulation" domain in the regulation of the m2 mAChR. In addition, the m4 mAChR subtype contains a Tyr at the corresponding position as the m2 subtype, and except for a substitution of Gln for the His immediately preceding the Tyr in m2, the amino acids surrounding the cytoplasmic tail Tyr of m4 mAChR are identical or conserved with those of m2 (Fig. 6). The m1 and m5 subtypes contain Trp in the corresponding position of m2 Tyr-459 (Fig. 6). Although a Trp substitution in m2 attenuated down-regulation, the differences in the surrounding sequence may create a down-regulation motif in the m1 and m5 mAChR. The only other Tyr present in the cytoplasmic tail of the mAChR subtypes is a Tyr residue in m3 and m5 carboxyl to m2 Tyr-459, but the sequence surrounding all these Tyr residues are dissimilar. There is also no apparent consen sus sequence surrounding the Tyr residues of m2 and the two Tyrs of β 3 AR shown to be involved in down-regulation.

In summary, the cytoplasmic tail tyrosine residue of the m2 mAChR is involved in agonist-induced down-regulation of the receptor, a process independent of short term agonist-induced sequestration. The m2 Tyr-459 residue is likely part of a motif necessary for lysosomal degradation of the m2 receptor, al-
though additional studies will be required for elucidating the
effect pathways involved in the down-regulation of the other
mAChR subtypes.

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