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Enhanced Coupling of M_1 Muscarinic Receptors to Activation of Phospholipase C upon Mutation of a Transposed Amino Acid Triplet Repeat

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The C-terminus ends of the second putative transmembrane domains of both M_1 and M_2 muscarinic receptors contain a triplet of amino acid residues consisting of leucine (L), tyrosine (Y) and threonine (T). This triplet is repeated as LYT-TYL in M_1 receptors at the interface between the second transmembrane domain and the first extracellular loop. Interestingly, however, it is repeated in a transposed fashion (LYT-LYT) in the sequence of M_2 receptors. In our previous work, we investigated the possible significance of this unique sequence diversity for determining the distinct differential receptor function at the two receptor subtypes. However, we found mutation of the LYTTYL sequence of M_1 receptors to the corresponding M_2 receptor LYTLYT sequence demonstrated markedly enhanced the stimulation of phosphoinositide (PI) hydrolysis by carbachol without a change in its coupling to increased cyclic AMP formation. In this work, thus, the enhanced stimulation of PI hydrolysis in the LYTLYT M_1 receptor mutant was further investigated. The stimulation of PI hydrolysis by carbachol was enhanced in the mutant M_1 receptor, and this change was not due to alterations in the rate of receptor desensitization or sequestration. The observed larger response to carbachol at mutant M_1 receptors was also not due to an artifact resulting from selection of CHO cells which express higher levels of G-proteins or phospholipase C.
Our data suggest that although the LYTTYL sequence in M_1 muscarinic receptors is not involved in determining receptor pharmacology, mutation of the sequence enhanced the coupling of M_1 receptors to the stimulation of phospholipase C.

Key Words: M_1 muscarinic receptors, Phospholipase C, Site-mutagenesis, Carbachol

INTRODUCTION

Genes encoding five distinct subtypes of mammalian muscarinic acetylcholine receptors have been molecularly cloned (Bonner et al., 1987; Peralta et al., 1987; Liao et al., 1989; Kashihara et al., 1992; Van Koppen et al., 1993). Non-mammalian muscarinic receptor genes have also been cloned and sequenced (Shapiro et al., 1989; Tietje et al., 1990).

Site-directed mutagenesis of the amino acid sequence of the different muscarinic receptors have resulted in significant advances in our knowledge regarding the importance of individual regions or single residues in the interaction of agonists and subtype-selective antagonists with muscarinic receptors, the coupling of the receptor to activation or inhibition of different second messenger pathways, and the role of specific regions in the process of agonist-induced receptor regulation (Maeda et al., 1990; Lechleiter et al., 1991a; 1991b; Hosey, 1992; Wess, 1993a; Brann et al., 1993).

We took special note of the intriguing repeat of the amino acid triplet leucine-tyrosine-threonine (LYT, using single letter amino acid designation) at or near the beginning of the first putative extracellular loop in case of the M_2 receptor (LYTLYT), and the existence of an inverted repeat of this triplet in the M_1 receptor

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sequence (LYTTYL) (Hulme et al., 1990). In our previous studies, we investigated the possible significance of these interesting sequence differences between M_1 and M_2 muscarinic receptors in terms of determining their pharmacological profile (Zhu et al., 1995; Lee et al., 1996a; Lee et al., 1996b). Mutation of the LYTTYL sequence of M_1 receptors to the corresponding M_2 receptor LYTLYT sequence, however, did not result in a significant change in the binding affinity of the agonist carbachol or in the affinity of the majority of a series of receptor antagonists which are able to discriminate between wild-type M_1 and M_2 receptors (Zhu et al., 1995). However, the LYTLYT M_1 receptor mutant demonstrated the markedly enhanced stimulation of phosphoinositides (PI) hydrolysis by carbachol (Lee et al., 1996a). Thus, in this work, we further investigated the enhanced stimulation of PI hydrolysis by carbachol at the LYTLYT m1 receptor mutant.

MATERIALS AND METHODS

Site-directed mutagenesis and cell culture procedures

Mutation in the DNA sequence was obtained using the Altered sites mutagenesis system (Promega). Primers used for mutagenesis were designed to alter two amino acids simultaneously in the sequence of M_1 muscarinic receptor. This resulted in the mutation of the LYTTYL sequence at positions 81~86 of the rat M_1 receptor into LYTLYT. Mutation was confirmed by dideoxy chain termination DNA sequencing. Wild-type and mutant receptors were stably expressed in Chinese hamster ovary (CHO) cells using the pCMV-3 mammalian expression vector according to the method of Zhu et al. (1994). Transfected CHO cells were grown in Dulbecco's modified Eagle medium containing 10% bovine calf serum and 0.05% geneticin. Cells were incubated at 37°C in 10% CO_2/90% humidified air and were used for experiments 4 days after culturing.

Radioligand binding experiments

Cells were collected and suspended in Krebs-Henseleit buffer (pH 7.4) consisting of (mM): NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 1.3; $NaHCO_3$, 25; glucose, 1.2 bubbled with 95% O_2/5% CO_2. Receptor concentration was determined by incubating intact cells with 0.01-4 nM [^{3}H]N-methylscopolamine ([^{3}H]NMS) for 1 hour at 37°C and nonspecific binding was measured in the presence of 2 μM atropine. Unbound radioactivity was separated by filtration through G/F filter (Zhu et al., 1994). Binding of the agonist carbachol was studied in cell membranes to avoid the effects of endogenous GTP (Nathanson, 1983). Cells were suspended in 20 mM Tris-HCl buffer/5 mM EGTA (pH 7.4) and homogenized by Polytron (23,000 rpm; 2×15 seconds). The homogenate was centrifuged at 750 *g* for 10 minutes and the obtained supernatant was centrifuged at 27,000 *g* for 30 minutes. Competition assays were performed in 20 mM Tris, 10 mM $MgCl_2$ (pH 7.4) and incubations were for 1 hour at 37°C.

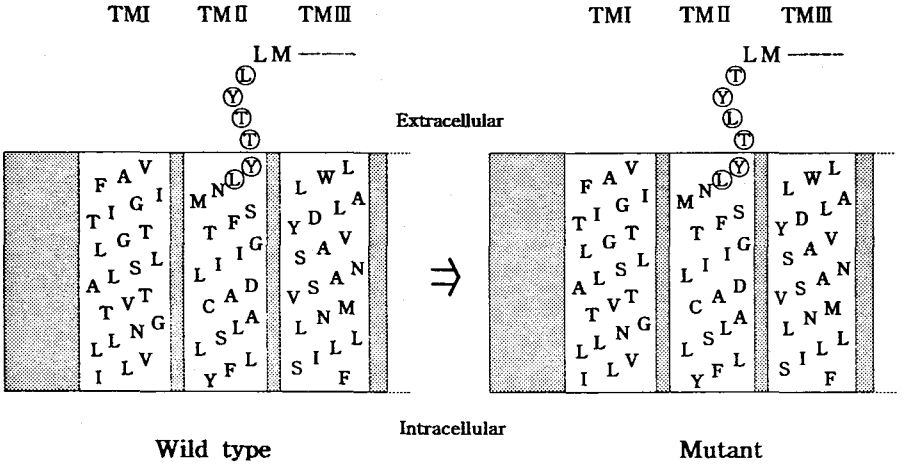

Fig. 1. Schematic representation of the mutated sequences in M_1 muscarinic receptors. Four transmembrane domains (TMIV-TMVII), three intracellular loops, three extracellular loops, C-terminal chain and N-terminal chain were omited in this figure because of insufficient space.

Assay of PI hydrolysis

Cells were labeled in oxygenated Krebs-Henseleit buffer with 10 μCi/ml of *myo*-[^{3}H]inositol (1 hour at 37°C). PI hydrolysis in presence of carbachol was continued for 1 hour at 37°C in Krebs-Henseleit buffer containing 10 mM LiCl. Total inositol phosphates were separated by ion-exchange chromatography using [^{14}C]inositol-1-phosphate as a standard as according to the method of Wang and El-Fakahany (1993).

Data analysis

The data are presented as the means±S.E.M. and statistical comparisons between different means were performed using Student's t-test and statistical significance was defined at the level of $P<0.05$.

Doseresponse curves were fitted according to a logistic fourparameter sigmoid model using the computer program GraphPad (ISI, Philadelphia). Competition curves of carbachol were better fitted in all cases to a twosite binding model ($P<0.05$) using the iterative fitting program LIGAND (Munson and Rodbard, 1980).

RESULTS

Receptor concentration in CHO cells expressed wild-type or mutant muscarinic receptors

We collected the single CHO cell expressed a similar number of either wild-type or mutant receptors in these particular experiments in order to preserve the stoichiometry between the number of receptors

Table 1. Receptor concentration in CHO cells expressed wild-type or mutant M_1 muscarinic receptors

Receptor type	B_{max} (fmol/mg protein)	K_D (pM)
Wild-type	648±61	259±15
Mutant	717±68	341±26

B_{max}: the maximal binding
K_D: the dissociation constant
Each value represents the mean±S.E.M. from five independent experiments.

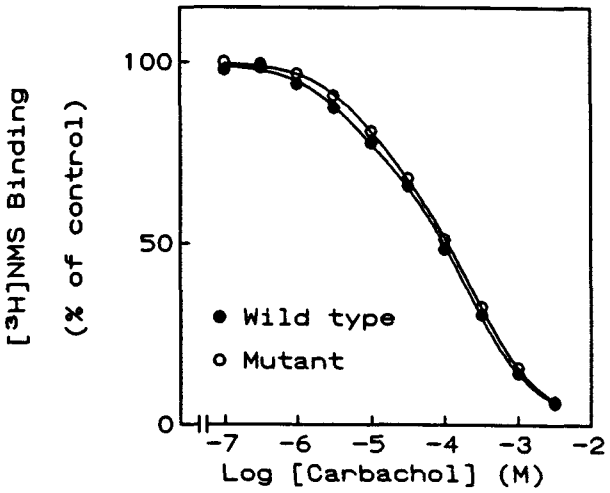

Fig. 2. Binding of carbachol to wild-type and mutant M_1 muscarinic receptors. CHO cell membranes were incubated with 0.2 nM [^{3}H]NMS in the absence or in the presence of increasing concentrations of carbachol. Incubation were in 20 mM Tris/10 mM $MgCl_2$ (pH 7.4) for 1 hour at 37°C. Each point is the mean±S.E.M. from 5 independent experiments.

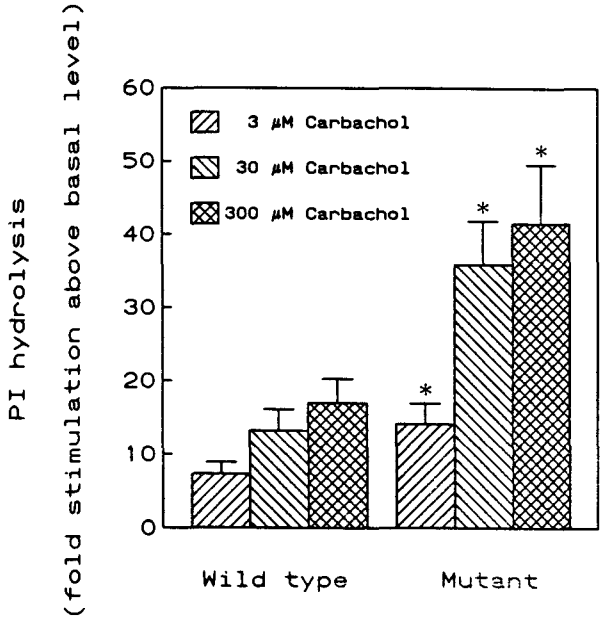

Fig. 3. Potentiation of M_1 receptor-receptor-mediated PI hydrolysis at the LYTLYT receptor mutant.
CHO cells which express a similar receptor number of wild-type and mutant M_1 receptors were incubated with or without increasing concentrations of carbachol for 60 minutes at 37°C in the presence of 10 mM LiCl. Data are presented as the means±S.E.M. of 6 independent experiments.
*$P<0.05$ as compared to the corresponding response at the wild-type receptor.

and G-proteins. The collected CHO cell was cultured and used for experiments. B_{max} values at wild-type and LYTLYT M_1 mutant receptors were 648±61 and 717±68 fmol/mg protein, respectively, with corresponding K_D values of 259±15 and 341±26 pM (Table 1).

Lack of effects of mutations in the first extracellular loop on agonist binding at M_1 muscarinic receptors

These experiments were performed in cell membranes to avoid the effects of endogenous GTP on agonist binding (Nathanson, 1983). Furthermore, it was important to use CHO cells which express a similar number of either wild-type or mutant receptors in these particular experiments in order to preserve the stoichiometry between the number of receptors and G-proteins. Competition curves of carbachol and [^{3}H]NMS were better fitted to a two-site as compared to a one-site binding model ($P<0.05$), both in case of their mutant counterparts. The K_D values of carbachol at its high and low-affinity binding sites at the wild-type m1 receptor were 5±1 μM (44±2% of receptors) and 150±8 μM, respectively. The LYTTYL→LYTLYT mutation in M_1 receptors did not result in any significant changes in the affinity of carbachol at its high and low-affinity binding sites or in the relative distribution of these sites as compared with the wild-type receptors (Fig. 2).

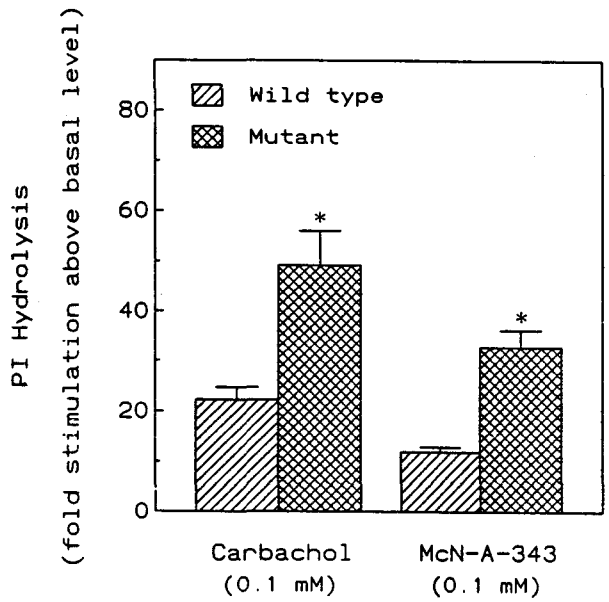

Fig. 4. Effects of the LYTTYL→LYTLYT mutation of the M_1 receptor on the PI response to the partial agonist McN-A-343. Receptor stimulation by either 0.1 mM McN-A-343 or 0.1 mM carbachol was continued for 60 minutes at 37°C in the presence of 10 mM LiCl. Data are the means±S.E.M. of 5 independent experiments. *$P<0.05$ as compared to the corresponding response at the wild-type receptor.

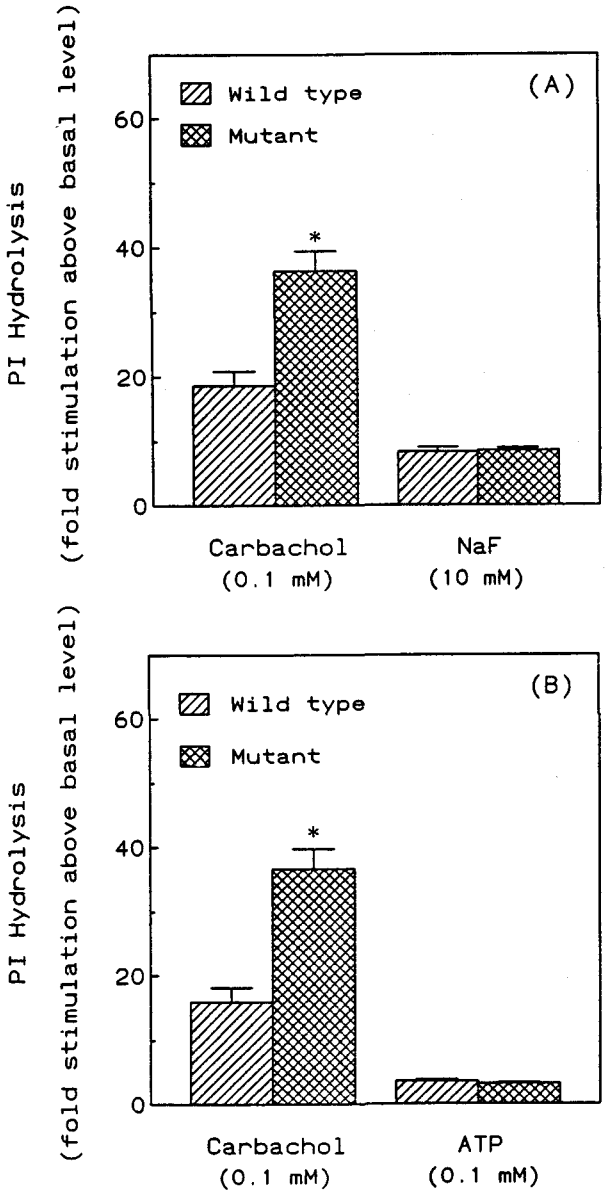

Fig. 5. Absence of changes of the PI response to NaF in CHO cells which express wild-type or mutant M_1 receptors. CHO cells which express either wild-type or mutant M_1 muscarinic receptors were activated in the presence of 10 mM LiCl with 10 mM NaF/25 μM $AlCl_3$ (A) or 0.1 mM ATP (B) for 60 minutes at 37°C. Carbachol (0.1 mM) was used as a control for comparison. The data presented are the means±S.E.M. of 5~6 independent experiments.

Effects of the LYTTYL→LYTLYT mutation on the coupling of m1 receptors to activation of PI hydrolysis

M_1 muscarinic receptors are preferentially coupled to stimulation of PI hydrolysis and their activation also increases cyclic AMP formation (Peralta et al., 1988; Wess, 1993b). CHO cells which express a similar number of wild-type and mutant M_1 muscarinic receptors were used for this experiment (637±69 and 728 ± 65 fmol/mg protein, respectively). Increment of PI hydrolysis by carbachol was markedly enhanced at the LYTLYT M_1 receptor mutant as compared to the wild-type receptor (Fig. 3). This is consistent with our prior study (Lee et al., 1996a). There was also a significant increase in the PI response to the M_1-selective partial agonist McN-A-343 (Wang and El-Fakahany, 1993) at the mutant M_1 receptor (Fig. 4). Basal levels of PI hydrolysis were not different in CHO cells which express wild-type or mutant receptors (3,534± 317 and 4,256±411 dpm/mg protein, respectively), although there was a slight increase in the level of uptake of [^{3}H]*myo*-inositol in cells transfected with the mutant receptor gene as compared to those which express the wild-type receptor (502±8 and 369±6 dpm/μg protein, respectively). This difference, however, did not appear to muscarinic agonists at the mutant receptor, since there were no significant differences in the response to direct activation of G-proteins by sodium fluoride or to stimulation of endogenous purinergic receptors by ATP in CHO cells which express wild-type or mutant M_1 receptors (Fig. 5).

Lack of effects of the LYTTYL→LYTLYT mutation in M_1 receptors on receptor desensitization or sequestration:

The time course of formation of inositol phosphates was compared at wild-type and mutant M_1 receptors in the absence of LiCl. Data shown in Fig. 6 demonstrate that the PI response at the LYTLYT M_1 receptor was markedly higher than that at the wild-type receptor at both short and long incubations. There was also an equal decrease in the binding of the cell-impermeable ligand [^{3}H]NMS to intact CHO cells which express wild-type or mutant M_1 receptors following incubation of cells with 1 mM carbachol (Fig. 7). Taken together, these results suggest that the enhanced response at mutant receptors is not due to differences in their ability to undergo desensitization or sequestration.

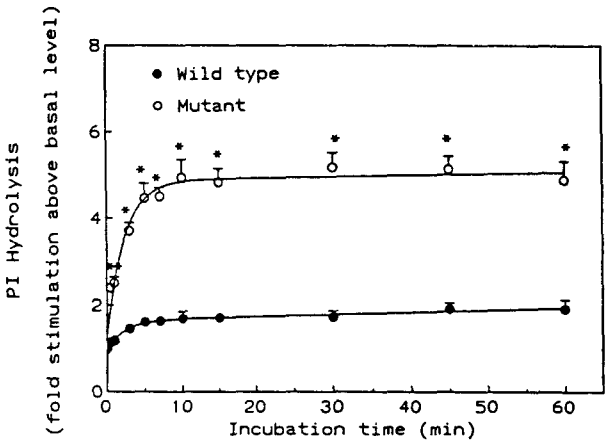

Fig. 6. Time course of carbachol-induced formation of inositol phosphates at wild-type and mutant M_1 receptors. The time course was studied upon stimulation with 0.1 mM carbachol in the absence of LiCl. Data shown are the means±S.E.M. of 4 independent experiments.

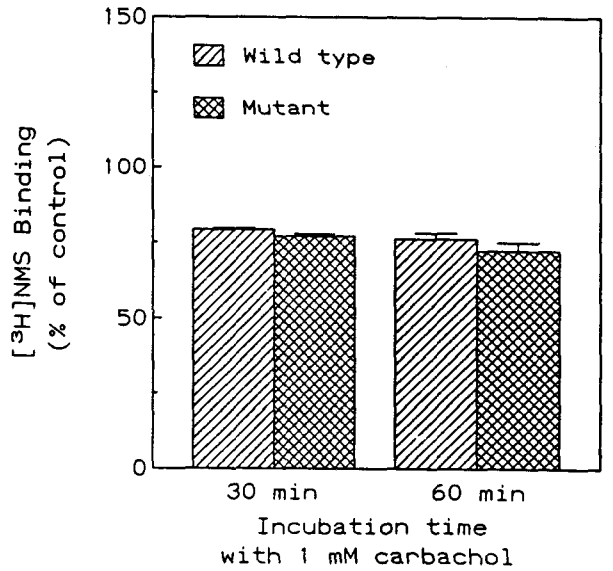

Fig. 7. Lack of differences in agonist-induced receptor sequestration at wild-type and mutant M_1 muscarinic receptors. CHO cells which express wild-type or mutant M_1 muscarinic receptors were incubated with 1 mM carbachol for 30 or 60 minutes, followed by washing. [^{3}H]NMS binding(1 nM) to intact cells was measured upon incubation for 3 hours at 15°C. Results shown are the means±S.E.M. of 4 independent experiments.

DISCUSSION

It has been suggested that several transmembrane

aspartate, tyrosine and threonine residues contribute to the interaction of agonists with muscarinic receptors (Fraser et al., 1989; Wess et al., 1991). We took special note of the intriguing repeat of the amino acid triplet leucine-tyrosine-threonine (LYT, using single letter amino acid designation) at or near the beginning of the first putative extracellular loop in case of the M_2 receptor (LYTLYT), and the existence of an inverted repeat of this triplet in the M_1 receptor sequence (LYTTYL) (Hulme et al., 1990).

In our previous studies, we investigated the pharmacological significance of the LYTTYL and the LYTLYT sequences which are located at or near the beginning of the putative first extracellular loops of M_1 and M_2 muscarinic receptors, respectively. We found that these interesting differences in sequence are not required for imparting selective interactions of antagonists or agonists at the two receptor subtypes (Zhu et al., 1995). Surprisingly, however, the LYTTYL→LYTLYT mutation markedly enhanced coupling of M_1 receptors to increased PI hydrolysis and Ca^{2+} signaling (Lee et al., 1996a). And, two tyrosine (Y) residues are important for receptor activation (Lee et al., 1996b). In this work, therefore, the enhanced stimulation of PI hydrolysis by carbachol at mutant M_1 receptor was further investigated.

The present mutation at M_1 receptor did not result in any significant changes in the affinity of binding of carbachol to the high- and low-affinity receptor conformations. There were also no changes in the proportion of receptors which exist in the two affinity states. This mutation, however, resulted in marked potentiation in the coupling of M_1 muscarinic receptors to stimulation of PI hydrolysis when wild-type and mutant muscarinic receptors were expressed at similar levels. These results are consistent with those of our prior study (Lee et al., 1996a). This phenomenon was specific to activation of muscarinic receptors, since there was no difference in the ability of sodium fluoride or ATP to stimulate PI hydrolysis in CHO cells which express wild-type or mutant M_1 muscarinic receptors. Thus, the observed larger response to carbachol at mutant M_1 receptors was not due to an artifact resulting from selection of CHO cells which express higher levels of G-proteins or phospholipase C.

Investigation of the possible mechanisms underlying the higher PI response at mutant M_1 receptors showed that it is likely not due to an alteration in the rate of receptor desensitization or sequestration. There is also evidence that the enhanced response might not be due to an increase in the affinity of the receptor-G protein complex, since there was no change in the characteristics of agonist interaction at the mutant receptors. It remains to be investigated in future experiments whether the agonist-stimulated mutant M_1 receptor possesses a higher efficacy in producing activation of G-protein, or whether the mutant receptor is able to recruit species of G-proteins which are not normally activated by the wild-type receptor.

Regardless of the underlying mechanisms, the LYTTYL→LYTLYT mutation in the M_1 receptor produces a supersensitive receptor in terms of its coupling to activation of phospholipase C. It has previously been shown that short amino acid stretches located both at the beginning and at the end of the third cytoplasmic loop play a more pronounced role in determining preference for various signaling pathways at the different subtypes of muscarinic receptors (Wess et al., 1990; Lechleiter et al., 1991a; 1991b). Therefore, it is possible that the repeat amino acid triplets located in the vicinity of the start of the first extracellular loop of the muscarinic receptor might influence the conformation of these or other cytoplasmic domains to modulate receptor-G protein interactions.

In conclusion, the LYTTYL→LYTLYT mutation in the M_1 receptor resulted in a supersensitive receptor in terms of its coupling to activation of phospholipase C. This receptor supersensitivity might be due to modulation of the mode of G-protein activation by the agonist-stimulated receptor.

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