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# Effects of Site-Mutagenesis of an Amino Acid Triplet Repeat at $M_1$ and $M_2$ Muscarinic Receptors on Receptor Function

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## ABSTRACT

Both $M_1$ and $M_2$ muscarinic receptors contain a triplet of amino acid residues consisting of leucine (L), tyrosine (Y) and threonine (T) at C-terminus ends of the second putative transmembrane domains. This triplet is repeated as LYT-LYT in $M_2$ receptors at the interface between the second transmembrane domain and the first extracellular loop. Interestingly, however, it is repeated in a transposed fashion (LYT-TYL) in the sequence of $M_1$ receptors. In this work, we employed site-directed mutagenesis to investigate the possible significance of this unique sequence diversity for determining the distinct differential cellular function at the two receptor subtypes. Mutation of the LYTTYL sequence of $M_1$ receptors to the corresponding $M_2$ receptor LYTLYT sequence did not result in a significant change in the binding affinity of the agonist carbachol. The reverse mutation at the $M_2$ receptor also did not modify agonist affinity. Surprisingly, the LYTLYT $M_1$ receptor mutant demonstrated markedly enhanced coupling to activation of phospholipase C without a change in its coupling to increased cyclic AMP formation. There was also an enhanced receptor sensitivity in transducing elevation of intracellular $Ca^{2+}$. On the other hand, the reverse LYTLYT→LYTTYL mutation in the $M_2$ receptor did not alter its coupling to inhibition of adenylate cyclase, but slightly enhanced its coupling to stimulation of phosphoinositide (PI) hydrolysis. Our data suggest that the LYTTYL/LYTLYT sequence differences between $M_1$ and $M_2$ muscarinic receptors are not important for specifying ligand binding and coupling of various subtypes of muscarinic receptors to different cellular signaling pathways although they might play a role in the modulation of muscarinic receptor coupling to PI hydrolysis.

**Key Words:** Muscarinic receptors, Site-mutagenesis, Phosphoinositide hydrolysis, Adenylate cyclase, 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). Hydropathicity analysis of the amino acid sequences of the products of these genes has resulted in the prediction that muscarinic receptors possess seven transmembrane domains which are interconnected by extracellular and intracellular loops (Hulme *et al.*, 1990). This motif is shared among other G-protein-coupled receptors (Savarese and Fraser, 1992).

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Systematic modifications 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, 1990; Wess, 1993; Brann *et al.*, 1993). Such modifications have been accomplished by site-directed mutagenesis or through the creation of chimeric structures in which segments of different receptors are combined.

Most of these structural modification studies have targeted conserved residues located in the putative transmembrane domains (Fraser *et al.*,. 1989; Wess *et al.*, 1990a) or stretches of sequences in the intracellular loops (Shapiro and Nathanson, 1989; Wess *et al.*, 1990a; 1990b; Moro *et al.*, 1993; Zhu *et al.*, 1994). Studies of the role of extracellular domains in receptor function have been limited for the most part to investigations of the potential role of the consensus glycosylation sites at the amino terminus (Van Koppen and Nathanson, 1990) and the possible existence of a disulfide bridge between the first and second extracellular loops (Savarses *et al.*, 1992). It is interesting, however, that there are extracellular sequences which are clearly particular to specific muscarinic receptor subtypes. For example, $M_2$ muscarinic receptors possess a unique block of acidic amino acids located in the third extracellular loop (Hulme *et al.*, 1990). It has recently been shown that this sequence is important for the interaction of allosteric antagonists ant $M_2$ receptors (Leppik *et al.*, 1994).

Upon comparing the sequences of $M_2$ and $M_1$ muscarinic receptors, 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, and the existence of an inverted repeat of this triplet in the $M_1$ receptor sequence (Hulme *et al.*, 1990). Thus, the sequences LYTLYT and LYTTYL are located at or close to the start of the first extracellular loop of $M_2$ and $M_1$ receptors, respectively (Fig. 1).

It is interesting that this sequence dichotomy appears to be associated with the categorization of muscarinic receptors into two major functional classes. Thus, there is marked sequence similarity in this region between $M_2$ and M4 receptors on one hand, and between $M_1$, M3 and M5 receptors on the other (allowing for conserved substitutions) (Hulme *et al.*, 1990). These two different functional classes of muscarinic receptors are preferentially coupled to inhibition of adenylate cyclase and stimulation of phosphoinositide (PI)[1] hydrolysis, respectively (Peralta *et al.*, 1988; Liao *et al.*, 1990; Richards, 1991; Caulfield, 1993; Mckinney, 1993). In this work, we investigated the possible significance of these interesting sequence differences between $M_1$ and $M_2$ muscarinic receptors in terms of determining their differential coupling to signal transduction pathways.

## MATERIALS AND METHODS

### Site-directed mutagenesis and cell culture procedures

Mutations in the DNA sequences were obtained using the Altered Sites mutagenesis system (Promega). Primers used for mutagenesis were designed to alter two amino acids simultaneously in the sequence of either the $M_1$ or the $M_2$ muscarinic receptor. This resulted in the mutation of the LYTTYL sequence at positions 81-86 of the rat $M_1$ receptor into LYTLYT, and mutation of the LYTLYT sequence of the rat $M_2$ receptor (located at position 79-84) into LYTTYL. Mutations were 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℃ in 10% $CO_2$/90% humidified air and were used for experiments 4

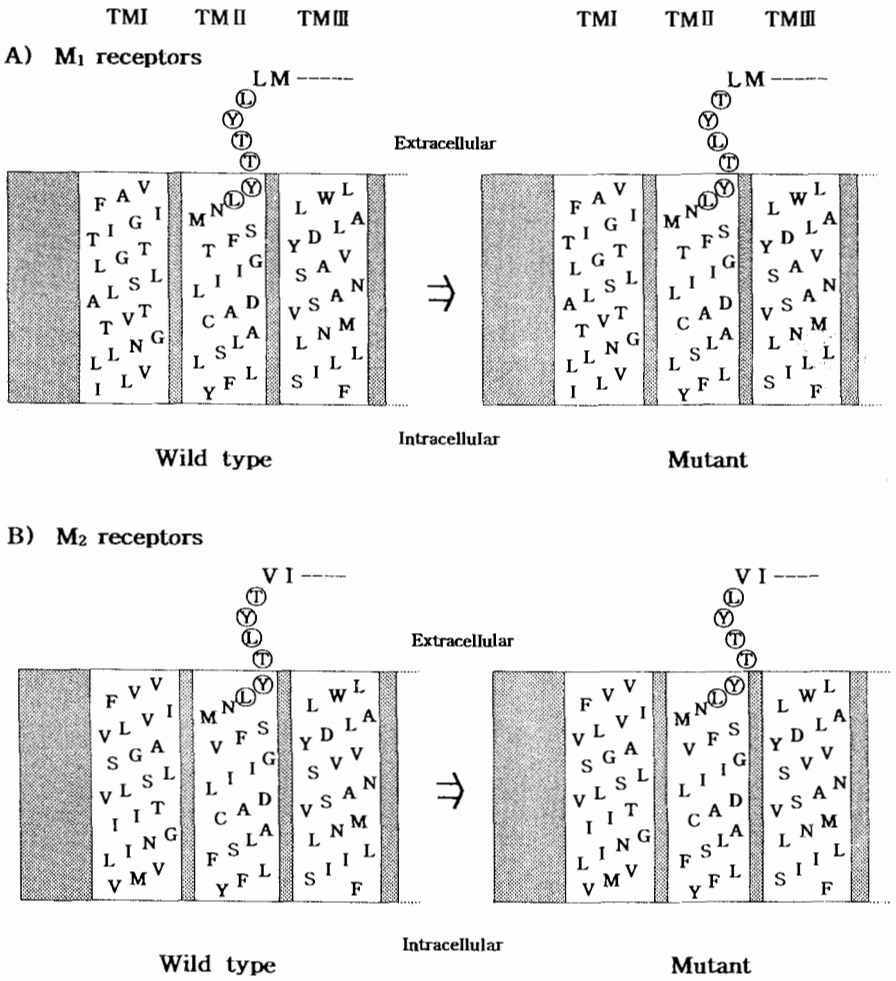


**Fig. 1.** Schematic representation of the mutated sequences in $M_1$ and $M_2$ muscarinic receptors. Four transmembrane domains (TMIV- TMVII), three intracellular loops, three extracellular loops, C-terminal chain and N-terminal chain were omited because of insufficient space.

days after subculture.

**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 $\mu$M atropine. Unbound radioactivity was separated by filtration through G/F filter.

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.

**Assay of PI hydrolysis**

Cells were labeled in oxygenated Krebs-Henseleit buffer with 10 $\mu$Ci/ml of myo-[$^3$H]inositol (1 hour at 37℃). PI hydrolysis in presence of carbachol was continued for 1 hour at 37℃ 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 described previously (Lee *et al.*, 1996).

**Assay of cyclic AMP formation**

CHO cells were suspended in oxygenated Krebs-Henseleit buffer and labeled with [$^3$H] adenine (10 $\mu$Ci/ml, 1 hour at 37℃), followed by washing and resuspension in buffer containing 1 mM isobutylmethylxanthine. Cyclic AMP formation was stimulated by 20 $\mu$M forskolin, in the absence or in the presence of increasing concentrations of carbachol. The reaction was stopped after 10 minutes at 37℃ and [$^3$H]cyclic AMP was isolated by ion-exchange chromatography using [$^{14}$C]cyclic AMP as a standard as according to the method of Wang and El-Fakahany (1993).

**Assay of changes in intracellular $Ca^{2+}$**

CHO cells were suspended in HEPES buffer of the following composition (mM): NaCl, 110; KCl, 5.4; $MgSO_4$, 1.8; glucose, 25; HEPES, 20 and sucrose, 58.4 (pH 7.4 and osmolality of 335-345 mOsmol). Cells were incubated with 5 $\mu$M fura-2/AM at 34℃ for 15 minutes followed by washing twice with buffer. Two ml of cell suspension ($\sim 10^6$ cell/ml) were transferred into a cuvette and maintained at 34℃ with continuous stirring. Agonist was added to the cuvette and the ratio of fluorescence intensity at excitation wavelengths of 340 and 380 nm at a fixed emission wavelength of 500 nm was measured in a spectrofluorometer (Perkin Elmer, Model LS50B). Calibration was performed using 10 $\mu$M ionomycin and 5 mM $MnCl_2$ for maximal and minimal fluorescence, respectively. Calculations of intracellular $Ca^{2+}$ concentrations were performed according to the method of Grynkiewics *et al.* (1985).

**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 $P < 0.05$. Dose-response curves were fitted according to a logistic four-parameter sigmoid model using the computer program GraphPad (ISI, Philadelphia). Competition curves of carbachol were better fitted in all cases to a two-site 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 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 637±69 and 728±65 fmol/mg protein, respectively. with corresponding $K_D$ values of 255±14 and 349±28 pM ($P < 0.05$). $B_{max}$ values at wild-type and LYTTYL $M_2$ mutant receptors were 78±2 and 72±3 fmol/mg protein, respectively. with corresponding $K_D$ values of 152±14 and 165±14 pM (Table 1).

**Effects of mutations in the first extracellular loop on agonist binding at $M_1$ and $M_2$ muscarinic receptors**

We investigated whether the LYTTYL/LYTLYT sequence difference between $M_1$ and $M_2$ muscarinic receptors might be responsible for the known higher binding affinity of muscarinic agonists to $M_2$ as compared to $M_1$ receptors (Lai *et al.*, 1992). These experiments were performed in cell membranes to avoid the effects of endogenous GTP on agonist binding (Nathanson, 1983). Competition curves of carbachol and [$^3$H]NMS were better fitted to a two-

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

| Receptor type | $B_{max}$ (fmol/mg protein) | $K_D$ (pM) |
|---|---|---|
| $M_1$ receptors | | |
| Wild-type | 637 ± 69 | 266 ± 14 |
| Mutant (LYTTYL → LYTLYT) | 728 ± 65 | 349 ± 28 |
| $M_2$ receptors | | |
| Wild-type | 78 ± 2 | 152 ± 14 |
| Mutant (LYTLYT → LYTTYL) | 72 ± 3 | 165 ± 14 |

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

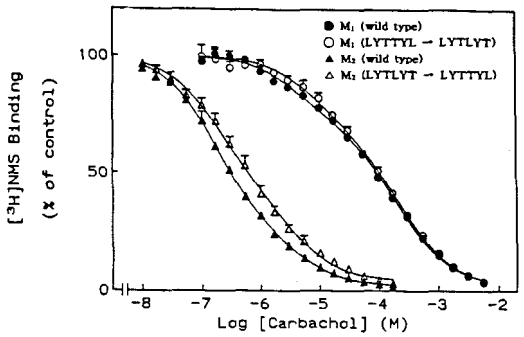


**Fig. 2.** Binding of carbachol to wild-type and mutant $M_1$ and $M_2$ muscarinic receptors. CHO cell membranes were incubated with 0.2 nM ($M_1$) or 0.4 nM ($M_2$) [$^3$H]NMS in the absence or in the presence of increasing concentrations of carbachol. Incubation were in 20 mM Tris/10 mM $MgCl_2$ (pH 7.4) for 1 hour at 37°C. Each point is the mean±S.E.M. from 5 independent experiments.

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 $M_1$ receptor were 5±1 μM (44±2% of receptors) and 150±8 μM, respectively. Carbachol interacted with the wild-type $M_2$ receptor with a markedly higher overall affinity and with a larger proportion of high-affinity binding sites as compared to its binding at $M_1$ receptors. Thus, the $K_D$ values of carbachol were 63±12 nM and 3±1 μM at the high-affinity and low-affinity binding sites at wild-type $M_2$ receptors, respectively.

**Table 2.** Effects of mutations at $M_1$ and $M_2$ muscarinic receptors on the binding of carbachol in CHO cell membranes

| Receptor type | $K_H$(μM) | $K_L$(μM) | %$R_H$ |
|---|---|---|---|
| $M_1$ receptors | | | |
| Wild-type | 5.4 ± 0.8 | 150 ± 8 | 44 ± 2 |
| Mutant | 10.5 ± 3.2 | 207 ± 34 | 48 ± 6 |
| $M_2$ receptors | | | |
| Wild-type | 0.06 ± 0.01 | 2.8 ± 0.9 | 83 ± 3 |
| Mutant | 0.11 ± 0.01 | 4.8 ± 0.7 | 80 ± 2 |

$K_H$: the equilibrium dissociation constants at the high-affinity receptor conformation
$K_L$: the equilibrium dissociation constants at the low-affinity receptor conformation
Each value represents the mean±S.E.M. from five independent experiments.

The high-affinity binding sites represented 83± 3% of the total receptors. The LYTTYL → LYTLYT mutation in $M_1$ receptors or the LYTLYT → LYTTYL mutation in the $M_2$ receptor 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 corresponding wild-type receptors (Fig. 2 and Table 2).

**Effects of the LYTTYL → LYTLYT mutation on the coupling of $M_1$ receptors to activation of PI hydrolysis and $Ca^{2+}$ signaling**

$M_1$ muscarinic receptors are preferentially

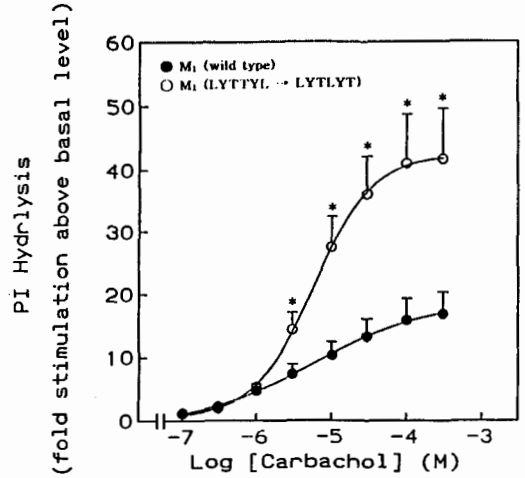


**Fig 3.** Potentiation of $M_1$ receptor-mediated PI hydrolysis at the LYTLYT receptor mutant. CHO cells which express a similar receptor number of wild-type and mutant $M_1$ receptors (see Table 1) were incubated with or without increasing concentrations of carbachol for 60 minutes at 37℃ in the presence of 10 mM LiCl. Data are presented as the means±S.E.M. of 6 independent experiments. *P < 0.05 as compared to the response at the wild-type receptor.

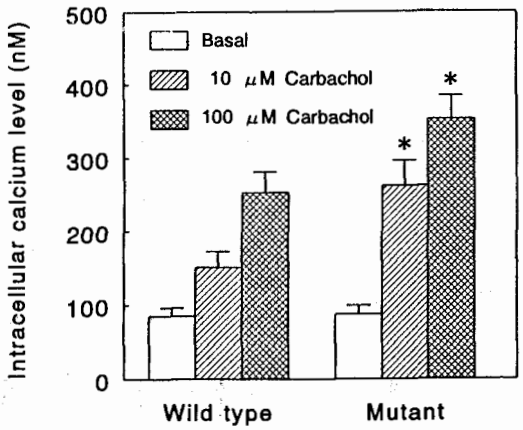


**Fig. 4.** Carbachol-induced increase in intracellular $Ca^{2+}$ at wild-type and mutant $M_1$ muscarinic receptors. Cells labeled with 5 μM fura-a/AM were stimulated with 10 or 100 μM carbachol, followed by measurement of the peak increase in intracellular $Ca^{2+}$ concentration. Data are presented as the means±S.E.M. of 9-12 independent experiments. *P<0.05 as compared to the corresponding value at the wild-type receptor.

coupled to stimulation of PI hydrolysis and their activation also increases cyclic AMP formation (Peralta *et al.*, 1988; Wess, 1993b). On the other hand, $M_2$ receptors are primarily coupled to inhibition of adenylate cyclase but are also weakly coupled to increased PI hydrolysis (Peralta *et al.*, 1988; McKinney, 1993). We investigated whether our targeted mutations at the first extracellular loop might result in a switch of the coupling of the two receptor subtypes to signal transduction pathways, or in modulation of the coupling of each receptor subtype to its preferred signaling pathway. CHO cells which express a similar number of wild-type and mutant $M_1$ muscarinic receptors were used. Unexpectedly, carbachol-induced enhancement of PI hydrolysis was markedly exaggerated at the LYTLYT $M_1$ receptor mutant as compared to the wild-type receptor (Fig. 3). This was reflected as a statistically significant increase in the maximal response to carbachol, without a change in its potency.

Basal levels of PI hydrolysis were not different in CHO cells which express wild-type or mutant receptors (3,500±310 and 4,300±400 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 (490±8 and 360±5 dpm/μg protein, respectively).

Increased agonist-induced PI hydrolysis at mutant $M_1$ receptors was accompanied by an enhancement of the increase in intracellular $Ca^{2+}$ concentration induced by 10 μM or 100 μM carbachol (Fig. 4). On the other hand, there was no effect of this mutation on agonist-induced stimulation of cyclic AMP formation at $M_1$ receptors (Fig. 5).

**Effects of the LYTLYT → LYTTYL mutation of the $M_2$ receptor on its coupling to inhibition of adenylate cyclase and activation of phospholipase C**

The LYTLYT → LYTTYL mutation in the $M_2$ receptor did not result in any significant chang-

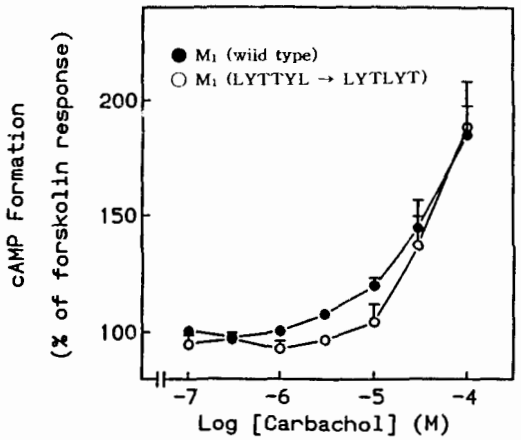


Fig. 5. Agonist-induced stimulation of cyclic AMP formation at the wild-type and the LYTLYT mutant $M_1$ muscarinic receptor. CHO cells which express wild-type or mutant $M_1$ muscarinic receptors were incubated with 20 $\mu$M forskolin in the absence or in the presence of increasing concentrations of carbachol. Incubations were for 10 minutes at 37℃. Each point is the mean±S.E.M. obtained in 3-4 independent experiments.

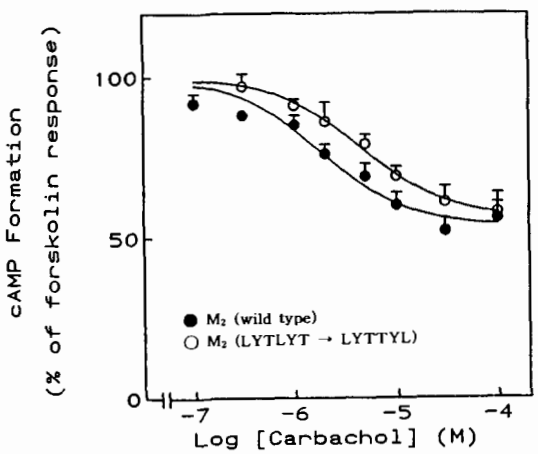


Fig. 6. Carbachol-induced inhibition of cyclic AMP formation at wild and LYTTYL mutant $M_2$ receptors. CHO cells labeled with [$^3$H]adenine were incubated with 20 $\mu$M forskolin in the absence and in the presence of increasing concentrations of carbachol for 10 minutes at 37℃. Data shown are the means±S.E.M. of 5 independent experiments.

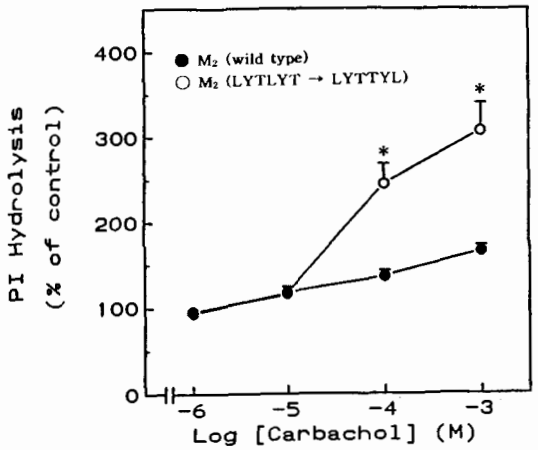


Fig. 7. Agonist-induced stimulation of PI hydrolysis at the wild-type and the LYTTYL mutant $M_2$ muscarinic receptor. PI hydrolysis was stimulated by increasing concentrations of carbachol in CHO cells which express wild-type or mutant $M_2$ muscarinic receptors. Incubations were for 60 minutes at 37℃ in the presence of 10 mM LiCl. The data are presented as the means±S.E.M. from 3 independent experiments. Basal PI hydrolysis averaged 1670 ±140 and 1280±80 dpm/$10^6$ cells in case of wild-type and mutant receptors, respectively, with corresponding values of 260,000±10,000 and 270,000±17,000 dpm/$10^6$ cells for [$^3$H]inositol uptake.
*$P<0.05$ as compared to the corresponding value at the wild-type receptor.

es in its coupling to inhibition of adenylate cyclase, either in terms of the maximal response to carbachol or in its potency at eliciting the response (Fig. 6). There was, however, an enhancement of coupling of $M_2$ receptors to increased PI hydrolysis at an equal level of receptor expression (78±2 and 72±3 fmol/mg protein in case of wild-type and mutant receptors, respectively). Thus, while 1 mM carbachol resulted in increased PI hydrolysis by 1.7 fold above basal level at the wild-type $M_2$ receptors, it elicited a 3.1-fold stimulation at the LYTTYL receptor mutant (Fig. 7).

## DISCUSSION

In this work we investigated the pharmaco-

logical 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.

Although 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), the LYTTYL → LYTLYT mutation markedly enhanced coupling of $M_1$ receptors to increased PI hydrolysis and $Ca^{2+}$ signaling, while the reverse mutation in the sequence of the $M_2$ receptor did not alter its coupling to inhibition of adenylate cyclase.

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). An interesting and well established phenomenon in the pharmacology of muscarinic receptor subtypes is that agonists bind to $M_2$ muscarinic receptors with significantly higher affinity as compared to their interaction at $M_1$ receptors (Lai *et al.*, 1992). There is evidence that unique sequences which are located at the amino and carboxy termini of the putative third cytoplasmic loop play a role in the determination of the magnitude of agonist binding affinity at various muscarinic receptor subtypes (Wess *et al.*, 1990b). These regions are the same ones which are responsible for specifying the interaction between different subtypes of muscarinic receptors and various G-proteins (Wess *et al.*, 1990b; Wess, 1993a; Hedin *et al.*, 1993). The present mutations did not result in any significant changes in the affinity of binding of carbachol to the high- and low-affinity receptor conformations at either $M_1$ or $M_2$ receptors. There were also no changes in the proportion of receptors which exist in the two affinity states. Thus, the LYTTYL-LYTLYT sequence difference between $M_1$ and $M_2$ muscarinic receptors does not appear to be relevant for the differential agonist binding at these two receptor subtypes.

It is generally accepted that $M_1$ muscarinic receptors are preferentially coupled to enhanced PI hydrolysis (Richards, 1991). Activation of these receptors also results in increased cellular cyclic AMP levels (Richards, 1991). On the other hand, $M_2$ muscarinic receptors efficiently transduce inhibition of adenylate cyclase, but are also weakly coupled to stimulation of PI hydrolysis (Peralta *et al.*, 1988). We therefore investigated whether the existence of the LYTTYL and LYTLYT sequences at the first extracellular loops of $M_1$ and $M_2$ receptors, respectively, is related to any extent to their specialized cellular functions. Mutation of the LYTTYL sequence of the $M_1$ receptor into LYTLYT did not reveal inhibition of adenylate cyclase, since carbachol still induced an equal increase in cyclic AMP formation at both wild-type and mutant $M_1$ receptors. 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. A second functional consequence of this receptor supersensitivity was demonstrated by the potentiation of the effects of submaximal carbachol concentrations in elevating intracellular $Ca^{2+}$.

The mutant LYTLYT → LYTTYL $M_2$ receptor remained fully coupled to inhibition of cyclic AMP formation to the same maximal level as compared with the wild-type receptor, without a change in agonist potency. On the other hand, this mutation resulted in an enhancement of coupling of $M_2$ receptors to activation of phospholipase C. The enhanced response was still much lower than that exhibited at the wild-type $M_1$ receptor. It should be borne in mind, however, that the low level of expression of $M_2$ receptors in CHO cells ($< 100$ fmol/mg protein, both in case of wild-type and mutant receptors) might mask the detection of a higher efficiency of coupling to PI hydrolysis at the mutant receptor.

Taken together, these data suggest that the LYTLYT → LYTTYL and the LYTLYT → LYTTYL mutations in the $M_2$ and the $M_1$ receptor sequences, respectively, result in positive modulation of their coupling to activation of phospholipase C, rather than in a switching of preferential receptor function. 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 pro-

nounced role in determining preference for various signaling pathways at the different subtypes of muscarinic receptors (Wess *et al.*, 1990b; 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, mutation of the LYTTYL sequence of $M_1$ receptors to LYTLYT and the opposite mutation in the $M_2$ receptor sequence did not result in switching of receptor function, although it produced a slight potentiation of coupling of $M_2$ receptors to stimulation of PI hydrolysis. On the other hand, the LYTTYL → LYTLYT mutation in the $M_1$ receptor resulted in a supersensitive receptor in terms of its coupling to activation of phospholipase C.

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=국문초록=

# $M_1$과 $M_2$ 무스카린성 수용체에서 아미노산 Triplet Repeat의 Site-Mutagenesis가 수용체기능에 미치는 영향

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$M_1$과 $M_2$ 무스카린성 수용체의 두 번째 transmembrane domain의 C-말단에는 leucine(L), tyrosine(Y), threonine(T)로 구성된 3중체(triplet)가 있다. 이 3중체는 $M_2$ 무스카린성 수용체에서는 두 번째 transmembrane domain과 첫 번째 세포외 고리사이의 연접부위에서 LYT-LYT의 반복구조로 존재하며 $M_1$ 무스카린성 수용체에서는 흥미롭게도 LYT-TYL의 역상구조로 존재한다. 본 연구에서는 site-directed mutagenesis방법을 사용하여 이와 같은 특이한 구조적 차이가 두 subtype의 수용체의 기능상 차이와 관련한 역할을 가지고 있는지를 확인하고자 하였다. $M_1$ 수용체에서는 LYTTYL서열을 $M_2$ 수용체의 서열에 해당하는 LYTLYT로 mutation시켰으며 $M_2$ 수용체에서는 LYTLYT8서열을 $M_1$ 수용체의 서열에 해당하는 LYTTYL로 mutation시켰다. 이와같은 mutation은 $M_1$과 $M_2$ 수용체에서 효능제 carbachol의 수용체 결합친화력에 유의한 변화를 주지 않았다. 또한 $M_1$ 수용체에서의 mutation은 cyclic AMP 증가작용에 대한 coupling은 변화시키지 않고 phosphoinositides (PI) hydrolysis 촉진작용과 세포내 $Ca^{2+}$ 농도 상승을 현저히 증가시켰다. 또한 $M_2$ 수용체에서의 mutation은 adenylate cyclase 억제에 대한 coupling은 변화시키지 않고 PI hydrolysis 촉진을 약간 증가 시켰다.

이상의 결과는 $M_1$과 $M_2$ 수용체에서 LYTTYL/LYTLYT 아미노산 서열의 차이는 두 수용체의 PI hydrolysis에 대한 coupling을 조절하는 역할을 하지만, 두 수용체 사이에서 ligand 결합과 신호전달계의 차이를 구분하는데 중요한 역할을 하지는 않는다.