

Location of a region of the muscarinic acetylcholine receptor involved in selective effector coupling

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Chimaeric muscarinic acetylcholine receptors (mAChR) in which corresponding portions of mAChR I and mAChR II are replaced with each other have been produced in *Xenopus* oocytes by expression of cDNA constructs encoding them. Functional analysis of the chimaeric mAChRs indicates that a region mostly comprising the putative cytoplasmic portion between the proposed transmembrane segments V and VI is involved in selective coupling of mAChR I and mAChR II with different effector systems. In contrast, the exchange of this region between mAChR I and mAChR II does not significantly affect the antagonist binding properties of the two mAChR subtypes.

Muscarinic acetylcholine receptor subtype; Chimaeric receptor; cDNA expression; Effector coupling; Selective antagonist; (*Xenopus* oocyte)

1. INTRODUCTION

The muscarinic acetylcholine receptor mediates a variety of cellular responses through the action of guanine nucleotide-binding regulatory proteins (G-proteins) [1]. Accumulating evidence indicates that molecularly distinct mAChR subtypes are selectively coupled with different effector systems, albeit not exclusively. Expression in *Xenopus* oocytes of cloned DNAs encoding four individual mAChR subtypes has revealed that mAChR I and mAChR III mediate activation of a Ca^{2+} -dependent Cl^- current, whereas mAChR II and mAChR IV principally induce activation of Na^+ and K^+ currents in a Ca^{2+} -independent manner [2–4]. Expression studies in mammalian cells have further shown that mAChR I and mAChR III are coupled efficiently with phosphoinositide hydrolysis [5–7], intracellular Ca^{2+} release [8], ac-

tivation of Ca^{2+} -dependent K^+ currents [5,8,9] and inhibition of the M-current [5,8], whereas mAChR II and mAChR IV are linked preferentially with adenylate cyclase inhibition [6,10]. It has also been revealed that the sensitivity to and the binding affinity for agonist of mAChR III are about one order of magnitude higher than those of mAChR I [4,8]. Moreover, different sensitivities to pertussis toxin of the cellular responses mediated by individual mAChR subtypes suggest that the response induced by mAChR I or mAChR III and that induced by mAChR II or mAChR IV occur through distinct G-proteins [5,10,11]. The antagonist binding properties of individual mAChR subtypes expressed in *Xenopus* oocytes [2,3,12] show that mAChR I, mAChR II and mAChR III correspond most closely to the pharmacologically defined M_1 (I), M_2 cardiac (II) and M_2 glandular (III) subtypes [13–15], respectively. This, together with the differential tissue distribution of the mRNAs encoding the individual mAChR species [2,16–19], indicates that the mAChR heterogeneity in tissues with respect to antagonist binding is attributable to the presence of distinct mAChR gene products by themselves or in various combinations.

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Abbreviations: mAChR, muscarinic acetylcholine receptor; ACh, acetylcholine; NMS, *N*-methylscopolamine; QNB, quinuclidinyl benzilate

The present investigation has been designed to localize the region of the mAChR molecules responsible for selective coupling with different effector systems. For this purpose, chimaeric mAChR molecules with different combinations of mAChR I and mAChR II have been produced in *Xenopus* oocytes by expression of the corresponding cDNAs and have been compared with the parental mAChRs with respect to acetylcholine (ACh)-induced current responses and antagonist binding properties.

2. MATERIALS AND METHODS

cDNAs encoding chimaeric mAChRs were constructed as follows. The 2.5-kilobasepair (kb) *PvuI/SacII* fragment and the 61-basepair (bp) *SacII/FokI* fragment from the plasmid pSPBM1 [3], the ~4.2-kb *DraII/PvuI* fragment from the plasmid pSPHM45 [3] and the synthetic oligodeoxyribonucleotide

5'-GGACCATCTTGGCTATTCTGCTAGCTTTCATCATCACTTGG-3'
3'-GTAGAACCGATAAGACGATCGAAAGTAGTAGTGAACCCGG-5'

(prepared with an automatic DNA synthesizer, Applied Biosystems) were ligated to yield the plasmid pSPMC2. The 166-bp *NsiI/XbaI* fragment from pSPHM45, the 542-bp *BstXI/BglII* fragment from pSPBM1 and the synthetic oligodeoxyribonucleotide

5'-CCGGGAAAAGAAAGTGACGAGGACGCTGAGCGCCATCCTG-3'
3'-CTTTCCTTCTACTGGTCCTGCGACTCGCGGTA-5'

were ligated and cleaved by *NsiI* and *BglII*. The resulting 748-bp fragment was ligated with the 2.5-kb *PvuI/NsiI* fragment from pSPHM45 and the ~2.5-kb *BglII/PvuI* fragment from pSPBM1 to yield the plasmid pSPMC4. The 377-bp *Sau3AI/NsiI* fragment from pSPHM45 and the synthetic oligodeoxyribonucleotide

5'-CATGGCCGCCTTCTACCTCCCTGT-3'
3'-CGGCGGAAGATGGAGGGACACTAG-5'

were ligated and cleaved by *NsiI*. The resulting 401-bp fragment was ligated with the ~5.8-kb *NsiI/HindIII* fragment from pSPHM45 and the 756-bp *HindIII/NcoI* fragment from pSPBM1 to yield the plasmid pSPMC7. The 376-bp *XbaI/SacII* fragment from pSPBM1 and the synthetic oligodeoxyribonucleotide

5'-GATCGTCATGTGCACACTCTACTGGCCCATCTACGGGAGACAGAGAACCGGGC-3'
3'-CAGTACACGTGTGAGATGACCGGTAGATGGCCCTCTGTCTTGGCCCGGGCC-5'

were ligated and cleaved by *SacII*. The resulting 430-bp fragment was ligated with the ~3.1-kb *SacII/PvuI* fragment from pSPBM1 and the 2.0-kb *PvuI/BclII* fragment from pSPHM45

to yield the plasmid pSPMC8. The 1.4-kb *SphI/NsiI* fragment from pSPMC7 and the ~4.2-kb *NsiI/SphI* fragment from pSPMC4 were ligated to yield the plasmid pSPMC9. The plasmid pmACR84 [2] was cleaved by *MstII*, treated with *T₄* DNA polymerase in the presence of the four deoxyribonucleoside triphosphates (blunt-ended), ligated with the synthetic *EcoRI* linker 5'-CCGGAATTCCGG-3' and cleaved by *EcoRI* and *BglII*. The resulting 1.7-kb *EcoRI/BglII* fragment was ligated with the ~0.9-kb *BglII/PvuII* fragment from pmACR84 and the 3.0-kb *SmaI/EcoRI* fragment from the plasmid pSP65 [20] to yield the plasmid pSPM26. pSPMC9 was cleaved by *MstII*, blunt-ended, ligated with the synthetic *EcoRI* linker and cleaved by *EcoRI* and *XbaI*. The resulting ~2.6-kb *EcoRI/XbaI* fragment was ligated with the 3.0-kb *XbaI/EcoRI* fragment from pSPM26 to yield the plasmid pSPMC9-1, which contained the same cDNA insert as pSPMC9, except that a portion of the 5'-noncoding region (nucleotide residues -167 to -74) was deleted. The 4.1-kb *XbaI/SacII* fragment from pSPMC8 and the ~2.7-kb *SacII/XbaI* fragment from pSPMC2 were ligated to yield the plasmid pSPMC10. All the constructs were confirmed by sequencing the regions derived from the synthetic oligodeoxyribonucleotides according to [21,22] and by restriction endonuclease analysis.

mRNAs specific for the chimaeric mAChRs MC2, MC4, MC7, MC8, MC9 and MC10 were synthesized by transcription in vitro [20,23] using *XbaI*-cleaved pSPMC2, pSPMC4, pSPMC7, pSPMC8, pSPMC9-1 and pSPMC10 as templates, respectively, unless otherwise indicated. mRNAs specific for mAChR I and mAChR II were synthesized as in [3]. Each of the mRNAs was injected into *Xenopus laevis* oocytes. The mRNA concentration used for injection was 10 ng/ μ l for MC10, 20 ng/ μ l for MC8 or 1 μ g/ μ l for the other chimaeric and parental mAChRs, unless otherwise indicated, and the average volume injected per oocyte was ~40 nl. The injected oocytes were incubated at 19°C for 2 days as in [2], except that the duration of incubation was 3 days for some of the measurements of (-)-[³H]quinclidinyl benzilate (QNB) binding in cell extracts and its displacement by unlabelled antagonists. These measurements were carried out as in [12]. The procedures used for the assay of (-)-[³H]*N*-methylscopolamine (NMS) binding on the cell surface and for the measurement of ACh-activated currents in Ringer's solution have been described in [2].

3. RESULTS AND DISCUSSION

Fig.1 schematically shows the structures of different chimaeric mAChRs in which corresponding portions of porcine mAChR I and mAChR II are replaced with each other. cDNAs encoding these chimaeric mAChRs were constructed and transcribed in vitro. Each of the resulting mRNAs was injected into *Xenopus* oocytes to produce the respective chimaeric mAChRs.

Fig.2 shows the ACh-induced current responses observed at -70 mV membrane potential in oocytes implanted with the different chimaeric mAChRs (C-G) and with the parental mAChR I

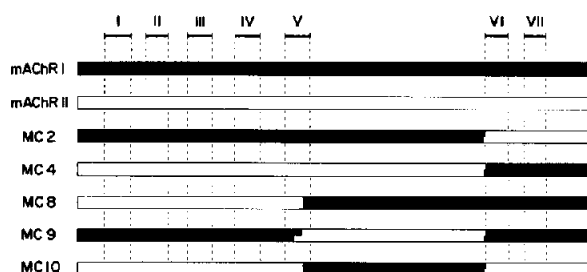
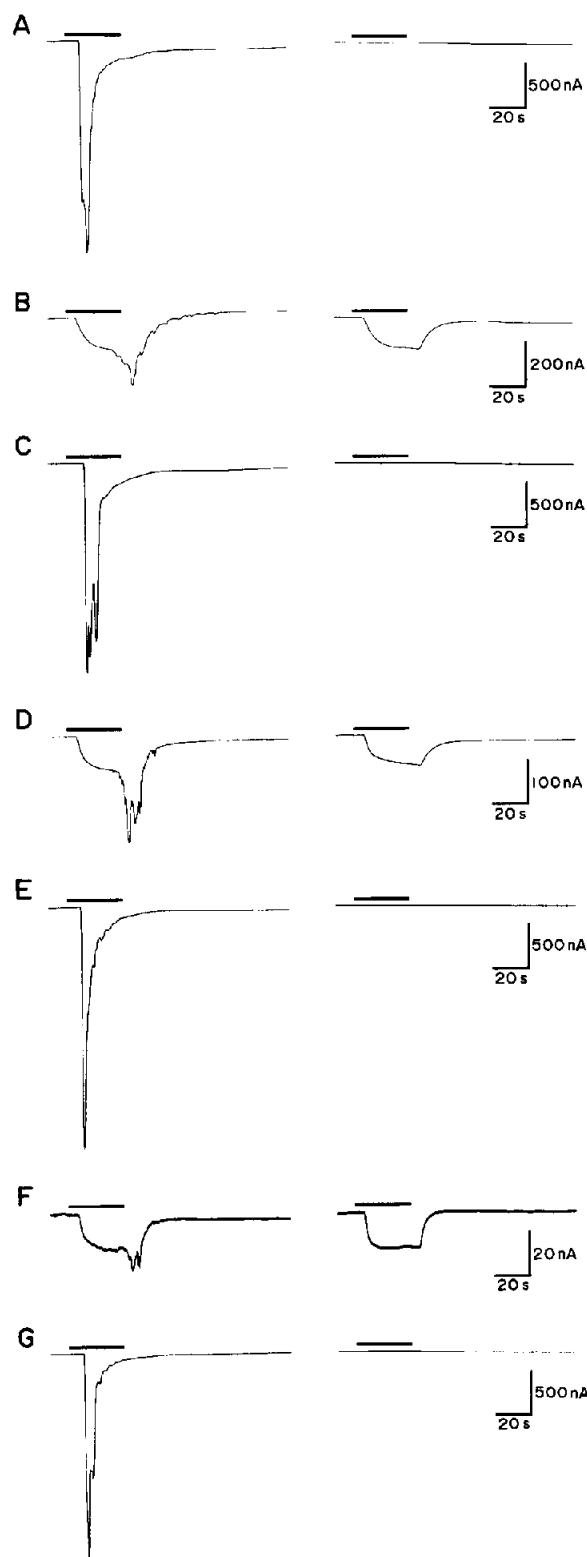


Fig.1. Structures of chimaeric mAChRs composed of porcine mAChR I (filled boxes) and mAChR II sequences (open boxes). Junctional sequences common to mAChR I and mAChR II are indicated by both boxes. The structures of chimaeric mAChRs are diagrammatically shown such that the putative transmembrane segments I–VII are aligned [2,16]; the fact that mAChR II differs from mAChR I in the lengths of the amino-terminal region preceding segment I (–2 amino acid residues), the region between segments V and VI (+24 residues) and the carboxy-terminal region following segment VII (–16 residues) is ignored. The compositions of the individual chimaeric mAChRs are as follows (the numbers in parentheses indicate amino acid numbers [2,16]; the junctional sequences common to mAChR I and mAChR II are represented by amino acid numbers of mAChR I): MC2, mAChR I (1–366) and mAChR II (389–466); MC4, mAChR II (1–386) and mAChR I (365–460); MC8, mAChR II (1–200) and mAChR I (203–460); MC9, mAChR I (1–201), mAChR II (200–386) and mAChR I (365–460); MC10, mAChR II (1–200), mAChR I (203–366) and mAChR II (389–466). In addition, MC7 composed of mAChR I (1–201) and mAChR II (200–466) was used (see the text).

(A) and mAChR II (B). The ACh-activated inward current mediated by the chimaeric mAChR MC2, MC8 or MC10 was oscillatory in nature (fig.2C,E,G, left traces) and was almost completely abolished by intracellular injection of the calcium-chelating agent EGTA (fig.2C,E,G, right traces). On the other hand, a typical ACh response mediated by the chimaeric mAChR MC4 or MC9

Fig.2. Effect of EGTA on ACh-activated currents in *Xenopus* oocytes injected with the mRNA specific for mAChR I (A), mAChR II (B), MC2 (C), MC4 (D), MC8 (E), MC9 (F) or MC10 (G). Whole-cell currents activated by bath application of 1 μ M ACh were recorded under voltage clamp at –70 mV membrane potential in Ringer's solution before (left traces) and after intracellular injection of EGTA (right traces). Inward current is downward. The duration of ACh application is indicated by bars without taking into account the dead-space time in the perfusion system (~6 s). EGTA was injected ionophoretically into the oocytes as in [3], except that the duration of injection was 10 min.



comprised an initial smooth inward current followed by an oscillatory component (fig.2D,F, left traces). The oscillatory current elicited by MC4 or MC9 varied in amplitude among the oocytes tested and was undetected in some of them. The smooth current evoked by MC4 or MC9 was virtually unaffected by intracellular injection of EGTA, whereas the oscillatory current disappeared after this treatment (fig.2D,F, right traces). Thus, MC2, MC8 and MC10, in which the putative cytoplasmic portion between the proposed transmembrane segments V and VI is derived from mAChR I, mediate an ACh response similar to that mediated by mAChR I (fig.2A; see also [2,3]). In contrast, MC4 and MC9, which share this portion with mAChR II, elicit an ACh response similar to that induced by mAChR II (fig.2B; see also [3]).

The average peak inward current activated by $1 \mu\text{M}$ ACh at -70 mV membrane potential was much larger in oocytes implanted with MC2, MC8 or MC10 than in oocytes implanted with MC4 or MC9 (fig.2 and table 1). The difference was more than two orders of magnitude in terms of average current per unit amount of $(-)-[{}^3\text{H}]\text{NMS}$ binding on the cell surface (an estimate of receptor density) (table 1). The average amplitudes of current response per unit amount of $(-)-[{}^3\text{H}]\text{NMS}$ binding observed for MC2, MC8 and MC10 were comparable to that of mAChR I, whereas similar values were obtained for MC4, MC9 and mAChR II (table 1). It is to be noted that the concentrations of the mRNAs injected ($10 \text{ ng}/\mu\text{l}$ to $1 \mu\text{g}/\mu\text{l}$) were chosen so that the $(-)-[{}^3\text{H}]\text{NMS}$ binding activity on the cell surface was within a range where the average current activated by $1 \mu\text{M}$ ACh was proportional to the binding activity; this proportionality was not examined for M9 which showed only a small current response even when the mRNA concentration was $1 \mu\text{g}/\mu\text{l}$. Oocytes injected with the mRNA encoding the chimaeric mAChR MC7 (see the legend to fig.1), which is almost a 'mirror image' of MC8, exhibited no detectable $(-)-[{}^3\text{H}]\text{NMS}$ binding activity on the cell surface and did not respond to ACh.

The ACh response mediated by MC9 and MC10 was further characterized. The latency of the ACh response in MC9-implanted oocytes was shorter than that in MC10-implanted oocytes, the former being attributable mostly to the dead-space time in the perfusion system ($\sim 6 \text{ s}$). The ACh response in

Table 1

Functional expression of chimaeric mAChRs in *Xenopus* oocytes

mAChR	$(-)-[{}^3\text{H}]\text{NMS}$ binding on cell surface (fmol per oocyte)	ACh-activated peak inward current (nA)	Current per unit amount of $(-)-[{}^3\text{H}]\text{NMS}$ binding (nA/fmol)
mAChR I	3.2 ± 2.0 (3)	2400 ± 1210 (32)	750
mAChR II	87.1 ± 37.3 (5)	210 ± 170 (28)	2.4
MC2	1.0 ± 0.2 (3)	1840 ± 760 (41)	1840
MC4	70.1 ± 29.1 (6)	163 ± 85 (30)	2.3
MC8	6.0 ± 0.8 (3)	2520 ± 1370 (20)	420
MC9	11.5 ± 7.1 (5)	22 ± 15 (20)	1.9
MC10	3.2 ± 1.2 (5)	2910 ± 1300 (33)	909

Data are given as means \pm SD; numbers in parentheses refer to the number of experiments for $(-)-[{}^3\text{H}]\text{NMS}$ binding activity or the number of oocytes for current measurements. Whole-cell currents activated by $1 \mu\text{M}$ ACh (bath-applied for 30 s) were recorded under voltage clamp at -70 mV membrane potential in Ringer's solution. Our detectable limits were $\sim 0.8 \text{ fmol}$ per oocyte for $(-)-[{}^3\text{H}]\text{NMS}$ binding activity and $\sim 8 \text{ nA}$ for ACh-activated current. Noninjected oocytes showed neither $(-)-[{}^3\text{H}]\text{NMS}$ binding nor ACh-activated current.

MC10-implanted oocytes occurred after an additional delay ($3 \pm 2 \text{ s}$, mean \pm SD, $n = 25$). The reversal potential of the oscillatory current elicited by MC10 in Ringer's solution was $-24 \pm 1 \text{ mV}$ ($n = 3$), which is close to the equilibrium potential of chloride ions in *Xenopus* oocytes [24]. The oscillatory current in MC9-implanted oocytes was reversed in polarity at a potential around -25 mV , whereas the reversal potential of the smooth current was around 10 mV . In MC9-implanted oocytes loaded with EGTA, the reversal potential of the smooth current was $9 \pm 1 \text{ mV}$ ($n = 7$). These results provide further evidence that the ACh response mediated by MC10 is similar to that mediated by mAChR I, whereas MC9 and mAChR II elicit a similar response.

The antagonist binding properties of the chimaeric mAChRs were examined in comparison with those of mAChR I and mAChR II, using oocyte extracts. The apparent dissociation constant (K_d) for $(-)-[{}^3\text{H}]\text{QNB}$ was similar for all the chimaeric and parental mAChRs (see the legend to fig.3), except for MC2 whose K_d value could not be evaluated because of its low binding activity. The apparent K_d values of MC4, MC8, MC9 and MC10

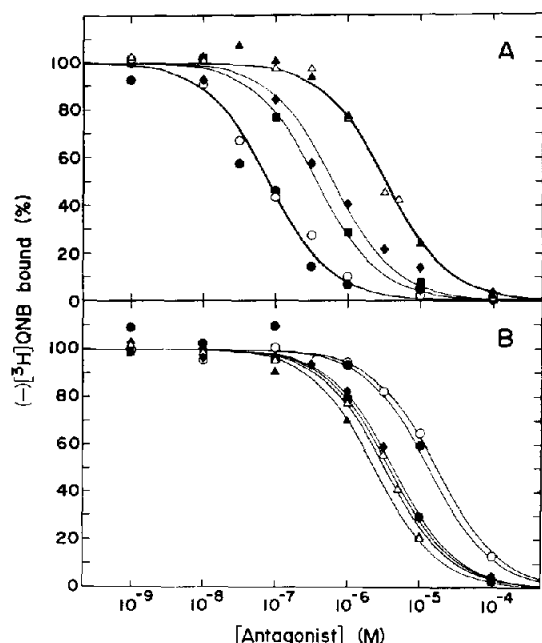


Fig.3. Effects of pirenzepine (A) and AF-DX 116 (B) on $(-)-[^3\text{H}]\text{QNB}$ binding in extracts from *Xenopus* oocytes injected with the mRNA specific for mACHR I (\circ), mACHR II (Δ), MC4 (\blacklozenge), MC8 (\blacksquare), MC9 (\bullet) or MC10 (\blacktriangle). The mRNA specific for MC9 was synthesized using *Xba*I-cleaved pSPMC9 as a template (see section 2). The concentration of each mRNA injected was $1\ \mu\text{g}/\mu\text{l}$. Data are from 3–5 experiments. Values for 100% and 0% binding were determined by measurements in the absence of antagonist and in the presence of $10\ \mu\text{M}$ atropine, respectively. The 0% values [89–210 dpm (\circ), 84–155 dpm (Δ), 81–144 dpm (\blacklozenge), 93–173 dpm (\blacksquare), 116–155 dpm (\bullet) or 127–195 dpm (\blacktriangle)] were 5–26% (\circ), 1–10% (Δ), 2–5% (\blacklozenge), 5–7% (\blacksquare), 23–38% (\bullet) or 0.7–3% (\blacktriangle) of the 100% values. The theoretical curves have been drawn by nonlinear least-squares analysis as in [2]. The IC_{50} values of pirenzepine and AF-DX 116 were 79.4 nM and 17.8 μM for mACHR I, 3.24 μM and 3.39 μM for mACHR II, 631 nM and 4.37 μM for MC4, 380 nM and 3.89 μM for MC8, 81.3 nM and 13.5 μM for MC9, and 3.31 μM and 2.45 μM for MC10, respectively. The K_d values for $(-)-[^3\text{H}]\text{QNB}$, obtained by Scatchard analysis from 3 experiments each, were $84 \pm 55\ \text{pM}$ for mACHR I [12], $130 \pm 17\ \text{pM}$ for mACHR II [12], $143 \pm 60\ \text{pM}$ for MC4, $100 \pm 64\ \text{pM}$ for MC8, $185 \pm 76\ \text{pM}$ for MC9 and $129 \pm 27\ \text{pM}$ for MC10. The K_d values for pirenzepine and AF-DX 116, calculated according to [25], were 13 nM and 2.9 μM for mACHR I, 0.75 μM and 0.79 μM for mACHR II, 0.16 μM and 1.1 μM for MC4, 72 nM and 0.73 μM for MC8, 24 nM and 4.1 μM for MC9, and 0.76 μM and 0.57 μM for MC10, respectively.

for the selective antagonists pirenzepine and AF-DX 116 were obtained by measuring displacement of $(-)-[^3\text{H}]\text{QNB}$ binding by increasing concentrations of the antagonists (fig.3). MC9 showed a

high affinity for pirenzepine ($K_d = 24\ \text{nM}$) similar to that of mACHR I ($K_d = 10\text{--}18\ \text{nM}$; see also [2,3,12]) and a low affinity for AF-DX 116 ($K_d = 4.1\ \mu\text{M}$) similar to that of mACHR I ($K_d = 1.8\text{--}2.9\ \mu\text{M}$; see also [3,12]). In contrast, MC10 showed a low affinity for pirenzepine ($K_d = 0.76\ \mu\text{M}$) similar to that of mACHR II ($K_d = 0.50\text{--}0.75\ \mu\text{M}$; see also [3,12]) and a high affinity for AF-DX 116 ($K_d = 0.57\ \mu\text{M}$) similar to that of mACHR II ($K_d = 0.36\text{--}0.79\ \mu\text{M}$; see also [3,12]). These results indicate that the region comprising mostly the putative cytoplasmic portion between the proposed transmembrane segments V and VI is not involved in binding to the selective antagonists. On the other hand, MC4 and MC8 showed an intermediate affinity for pirenzepine ($K_d = 0.072\text{--}0.16\ \mu\text{M}$) and an intermediate or high affinity for AF-DX 116 ($K_d = 0.73\text{--}1.1\ \mu\text{M}$). These results may indicate that both a region containing the putative transmembrane segments I–V and a region containing the putative transmembrane segments VI and VII are involved in antagonist binding. Affinity labelling and peptide mapping studies suggest that the ligand binding site of the mACHR is located deep within the membrane [26]. Similarly, an involvement of putative transmembrane segments in ligand binding has been reported for adrenergic receptors [27–29].

The present investigation reveals that the chimaeric mACHR MC10, in which only the segment composed of amino acid residues 203–364 of mACHR I is substituted for the corresponding segment of mACHR II (amino acid residues 201–386), mediates an ACh response indistinguishable from that mediated by mACHR I. Our results also show that the chimaeric mACHR MC9, which is almost a ‘mirror image’ of MC10 (amino acid residues 200–386 of mACHR II substituted for the corresponding residues 202–364 of mACHR I), evokes an ACh response indistinguishable from that induced by mACHR II. Thus, it is concluded that the region of the mACHR molecules comprising the carboxy-terminal third of the proposed transmembrane segment V and the following putative cytoplasmic portion before the proposed transmembrane segment VI contains a determinant of selective coupling with different effector systems. In contrast, the exchange of this region between mACHR I and mACHR II does not significantly affect the antagonist binding proper-

ties of the two mAChR subtypes. The amino acid sequence of the putative cytoplasmic portion between the proposed transmembrane segments V and VI is divergent among the mAChR subtypes [2,12,16–18,30,31]. However, some sequence homology is noted in this portion between mAChR I and mAChR III as well as between mAChR II and mAChR IV, particularly in the vicinity of segments V and VI. Furthermore, it has been shown that mAChR I and mAChR III are coupled with the same effector systems as are mAChR II and mAChR IV [2–10]. Thus, it seems reasonable to assume that a region adjacent to the carboxyl-terminus of segment V and a region adjacent to the amino-terminus of segment VI are responsible for selective coupling of mAChR subtypes with different effector systems, probably through interaction with distinct G-proteins.

Recent studies with chimaeras between the β_2 - and α_2 -adrenergic receptors have shown that the specificity for coupling with the stimulatory G-protein lies within a region extending from the amino-terminus of segment V to the carboxyl-terminus of segment VI [29]. It has also been reported that deletion of an amino-terminal or a carboxy-terminal sequence of the putative cytoplasmic portion between segments V and VI in the β_2 -adrenergic receptor leads to loss of stimulatory G-protein activation [32]. Furthermore, a single amino acid substitution in the portion between segments V and VI of rhodopsin has been shown to prevent activation of transducin [33]. Thus, the conclusion that a region mostly comprising the putative cytoplasmic portion between segments V and VI is involved in selective effector coupling may be generally valid for G-protein-coupled receptors.

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