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Distinct sequence elements control the specificity of G protein activation by muscarinic acetylcholine receptor subtypes

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Relatively little is understood concerning the mechanisms by which subtypes of receptors, G proteins and effector enzymes interact to transduce specific signals. Through expression of normal, hybrid and deletion mutant receptors in *Xenopus* oocytes, we determined the G protein coupling characteristics of the functionally distinct m2 and m3 muscarinic acetylcholine receptor (mAChR) subtypes and identified the critical receptor sequences responsible for G protein specificity. Activation of a pertussis toxin insensitive G protein pathway, leading to a rapid and transient release of intracellular Ca^{2+} characteristic of the m3 receptor, could be specified by the transfer of as few as nine amino acids from the m3 to the m2 receptor. In a reciprocal manner, transfer of no more than 21 residues from the m2 to the m3 receptor was sufficient to specify activation of a pertussis toxin sensitive G protein coupled to a slow and oscillatory Ca^{2+} release pathway typical of the m2 subtype. Notably, these critical residues occur within the same region of the third cytoplasmic domain of functionally distinct mAChR subtypes.

Key words: G protein selectivity/ion channels/muscarinic receptors

Introduction

One of the fundamental mechanisms of cell signaling involves the cascade of events initiated by a broad class of membrane-bound receptors and mediated by guanine nucleotide binding regulatory (G) proteins (Casey and Gilman, 1988; Neer and Clapham, 1988). The effector enzymes and ion channels regulated by this cascade modulate the intracellular concentrations of specific second messenger molecules which in turn regulate the activities of numerous target enzymes vital to cellular function. Although relatively few types of second messengers are generated, there is substantial diversity within the regulatory components themselves as evidenced by the isolation of molecular clones encoding structurally related subtypes of several different families of receptors, G proteins and effector enzymes (Dohlman *et al.*, 1987; Casey and Gilman, 1988; Nishizuka, 1989; Rhee *et al.*, 1989). Therefore, a central question regarding this form of transmembrane signaling concerns the mechanism by which individual receptor subtypes differentially interact with G proteins to regulate specific effector systems.

Muscarinic acetylcholine receptors (mAChRs) are a family of at least five distinct subtypes, designated m1 – m5, which interact with G proteins to regulate a variety of important physiological responses including neuronal excitation and inhibition, reduction of the rate and force of heart contraction, vasodilation and glandular secretion (Kubo *et al.*, 1986; Peralta *et al.*, 1987a,b; Bonner *et al.*, 1987; Nathanson, 1986). Biochemical analysis of the cellular effects mediated by mAChR subtypes expressed within various tissues or transfected mammalian cell lines indicates that mAChR subtypes can be divided into two functional classes. The m1, m3 and m5 subtypes are efficiently coupled to the stimulation of phosphoinositide (PI) hydrolysis via G protein activation of phospholipase C (PLC), while the m2 and m4 subtypes preferentially inhibit adenylyl cyclase activity and weakly stimulate PI hydrolysis through G proteins (Peralta *et al.*, 1988; Fukuda *et al.*, 1988). Hydrolysis of phosphatidylinositol 4,5-bisphosphate leads to the production of two important second messenger molecules: inositol 1,4,5-trisphosphate (IP_3), a mediator of intracellular calcium release, and diacylglycerol, an activator of protein kinase C (Berridge and Irvine, 1989). Inhibition of adenylyl cyclase results in a reduction in cAMP and cAMP dependent kinase activity within the cell (Gilman, 1987). Studies utilizing pertussis toxin (PTX), which catalyzes the ADP ribosylation of certain G proteins and abolishes their interactions with receptors, have also revealed differences in the abilities of mAChRs to interact with different G proteins. The large increase in PI hydrolysis mediated by the m1 or m3 subtypes is largely resistant to PTX, while the PI response and the inhibition of adenylyl cyclase evoked by the m2 or m4 subtypes are completely abolished by treatment with the toxin (Ashkenazi *et al.*, 1989).

Functional distinctions between mAChR subtypes in their abilities to activate the calcium second messenger pathway have also been observed in electrophysiological recordings of Ca^{2+} activated chloride currents (I-Cl) in *Xenopus* oocytes. Agonist stimulation of the m1 and m3 subtypes expressed in *Xenopus* oocytes results in a strong and rapid activation of I-Cl, while the m2 and m4 subtypes only weakly activate these channels (Fukuda *et al.*, 1987, 1988; Bujo *et al.*, 1988). We used these well-defined functional differences between mAChR subtypes as experimental criteria for identifying structural domains which control the specificity of interaction between receptor subtypes and G proteins. To determine regions of mAChR subtypes important for G protein recognition and activation, the G protein coupling properties of reciprocal hybrid receptors composed of domains derived from the functionally distinct m2 and m3 subtypes were assayed via transient expression in *Xenopus* oocytes. Surprisingly, our experiments demonstrated that a single contiguous domain of as few as nine amino acids was

sufficient to transfer m3-like coupling of PI hydrolysis and intracellular calcium mobilization to the m2 receptor subtype. In a reciprocal manner, a corresponding region of no more than 21 amino acids of the m2 receptor transferred into the m3 receptor resulted in m2-like G protein coupling properties. Site-directed mutagenesis of this region of the m3 receptor confirmed the importance of these sequences in G protein recognition and activation. These results suggest that analogous domains may be responsible for specifying the G protein coupling properties of related neurotransmitter and hormone receptors. The location of these domains, however, may vary between different classes of G protein-linked receptors.

Results

Construction and expression of hybrid receptors

The membrane topology of the five mAChR subtypes, similar to the visual rhodopsins, adrenergic and other neurotransmitter receptors, is predicted to consist of seven transmembrane domains with an extracellular amino terminus and an intracellular carboxy terminus (Figure 1) (Peralta *et al.*, 1987b). The high degree of sequence identity shared by all mAChR subtypes within the transmembrane domains and short connecting loop regions is consistent with acetylcholine binding to a site within a pocket created by insertion of the amphipathic, α-helical transmembrane domains into the lipid bilayer (Peralta *et al.*, 1987a). In contrast, the large intracellular domain connecting the fifth and sixth transmembrane segments differs extensively in size and primary sequence between the different mAChR subtypes. Notably, the two functional classes of mAChR subtypes share a limited degree of sequence identity even within this domain, particularly in regions immediately adjacent to the fifth and sixth transmembrane segments. These observations originally led to the suggestion that the large cytoplasmic domain may play an important role in determining the selectivity of G protein interaction by particular mAChR subtypes (Peralta *et al.*, 1987a).

To identify sequences which control G protein recognition by mAChRs, a series of hybrid receptors derived from the functionally distinct human m2 and m3 subtypes were

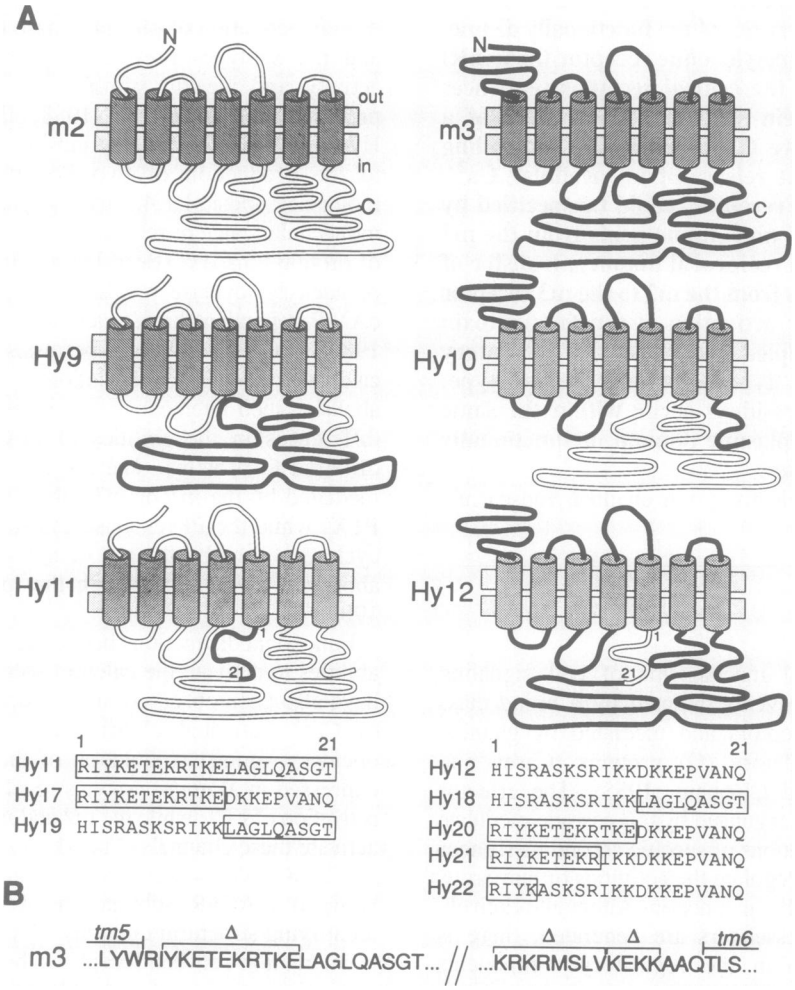

Fig. 1. Structure of parental, hybrid and deletion mutant mAChRs. (**A**) Parental m2 and m3 receptors are indicated by open and shaded lines, respectively. The amino (N) and carboxy (C) termini of each receptor are positioned on the extracellular (out) and intracellular (in) faces of the plasma membrane, respectively. The proposed transmembrane segments are depicted as cylinders. The actual sequences contained within the 21 amino acid region of hybrid receptors Hy11–22 are shown below; sequences within this region derived from the m3 receptor are boxed. Hy17 and Hy19 are identical to Hy11 and Hy18, Hy20, Hy21 and Hy22 are identical to Hy12 except for the composition of sequences within the 21 amino acid region as indicated. (**B**) The location of three deletion (Δ) mutations within the third cytoplasmic domain of the m3 subtype are indicated by brackets. The proposed cytoplasmic boundaries of the fifth and sixth transmembrane segments of the m3 subtype are indicated as tm5 and tm6, respectively.

constructed. Initially, reciprocal hybrid receptors were generated in which the entire cytoplasmic domain joining the fifth and sixth transmembrane segments was exchanged between the m2 and m3 subtypes (Figure 1). As described above, the two functional classes of mAChR subtypes share a limited degree of sequence conservation within the large cytoplasmic domain in regions immediately adjacent to the fifth and sixth transmembrane segments. Therefore, to test the role of more specific regions of the m2 and m3 subtypes in G protein recognition, pairs of reciprocal hybrid receptors were similarly constructed in which sequences adjacent to the fifth transmembrane segment were exchanged between the m2 and m3 subtypes (Figure 1).

To confirm that each hybrid receptor is capable of binding muscarinic ligands, antagonist and agonist binding studies were conducted following the stable transfection of human embryonic kidney cells with plasmids directing the expression of each receptor (Table I). Saturation binding experiments with the non-selective muscarinic antagonist *N*-methyl scopolamine ([^{3}H]NMS) revealed that each hybrid receptor displays high affinity binding with apparent dissociation constants (K_D) ranging from 210 to 390 pM. By comparison, the K_D for NMS binding with stably transfected cell lines expressing the parental m2 and m3 receptors is 260 and 340 pM, respectively (Table I). Next, the agonist binding properties of each hybrid receptor were determined by competition displacement experiments with [^{3}H]NMS and the acetylcholine analog carbachol. These experiments revealed that each hybrid receptor displays both high and low affinity sites for carbachol; two-site binding of agonist is a common property of the family of seven transmembrane receptors and is thought to reflect the interaction between these receptors and G proteins (Casey and Gilman, 1988). Hybrid mAChRs bound carbachol with K_D values for the high affinity site ranging from 0.3 to 5.0 μM, comparable with the carbachol K_D values of 5.1 and 4.8 μM displayed by the parental m2 and m3 receptors, respectively (Table I). These binding studies demonstrated that the alteration of putative intracellular protein sequences within these hybrid receptors did not hamper their ability to bind muscarinic agonists and antagonists.

Functional analysis in Xenopus oocytes identifies a receptor domain controlling the specificity of G protein activation

To identify protein sequences which control the specificity of G protein activation by mAChR subtypes, the ability of each hybrid receptor to stimulate I-Cl was compared with the parental m2 and m3 receptors within the *Xenopus* oocyte expression system. Previous studies have characterized the principal components of the Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes (Dascal *et al.*, 1984, 1985). These Cl^- currents are easily distinguished by their kinetics and, as we will show, by their PTX sensitivity. Briefly, we found that one of these components, the D1 current (first depolarizing, in the notation of Dascal *et al.*), was stimulated primarily by recombinant m3 receptors. The D1 inward current is characteristically fast, transient, and largely insensitive to PTX treatment (see below). A second major Cl^- current, the F (fluctuating) current, was found to be predominantly stimulated by m2 receptors. This current is characteristically slow, oscillatory, and, as we will show, very sensitive to PTX treatment. For clarity, we will herein refer to these currents as the m3-like and m2-like acetylcholine (ACh)-induced responses. More importantly, our data unequivocally attribute the m3- and m2-like current responses to separate and distinct G protein mediated pathways and allow us to identify receptor sequences controlling G protein specificity (see below).

ACh-induced responses were recorded using two electrode voltage clamp measurements 2 days after injection of individual oocytes with *in vitro* transcribed RNA encoding parental or hybrid mAChRs. The Cl^- currents reported here all reversed at the equilibrium potential for Cl^- (approximately −25 mV) and could be blocked by intracellular injections of EGTA (100 mM), identifying them as

Table I. Apparent dissociation constants (K_D) for the binding of antagonist (NMS) and agonist (carbachol) to parental, hybrid and deletion mutant mAChRs

mAChR transfected	[^{3}H]NMS sites/cell ($\times 10^{-3}$)	[^{3}H]NMS K_D (pM)	Carbachol		% H/T
			K_{DH} (μM)	K_{DL} (μM)	
m2	150 ± 12	260 ± 12	5.1 ± 0.2	–	100
m3	410 ± 9	340 ± 24	4.8 ± 2.1	25 ± 11	68
Hy9	260 ± 4	210 ± 13	0.8 ± 0.6	11 ± 5	53
Hy10	280 ± 12	240 ± 38	5.0 ± 2.2	56 ± 17	63
Hy11	82 ± 4	350 ± 50	0.7 ± 0.1	52 ± 8	94
Hy12	280 ± 61	210 ± 23	1.2 ± 0.8	120 ± 12	26
Hy17	400 ± 10	390 ± 30	0.7 ± 0.5	17 ± 2	25
Hy18	260 ± 6	210 ± 18	1.4 ± 0.9	36 ± 2	19
Hy19	380 ± 11	280 ± 27	5.0 ± 1.3	26 ± 18	86
Hy20	190 ± 2	210 ± 7	0.3 ± 0.1	42 ± 2	22
Hy21	35 ± 0.5	220 ± 12	3.8 ± 2.3	68 ± 3	33
Hy22	94 ± 6	230 ± 40	ND	ND	ND
m3ΔYKETEKRTKE	8 ± 0.6	150 ± 61	1.5 ± 0.8	25 ± 3	32
m3ΔKRKRMSLV	200 ± 11	530 ± 60	ND	ND	ND
m3ΔKEKK	140 ± 3	170 ± 19	ND	ND	ND

The saturation binding of [^{3}H]NMS and competitive binding of carbachol (means ± SEM) to intact cells are described in Materials and methods. All binding studies were done with massed populations of stably transfected cells except Hy11 cells which were derived by single cell cloning. K_{DH} and K_{DL} refer to the high and low affinity binding states for agonist, respectively. ND indicates agonist affinities not determined. % H/T refers to the amount of high affinity receptors as a percentage of the total number of receptors.

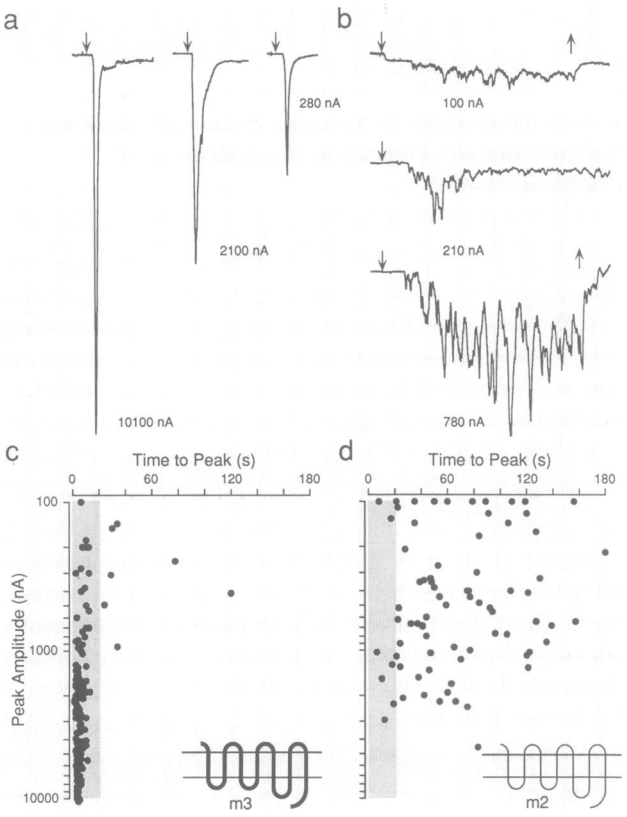

Fig. 2. Acetylcholine (ACh)-induced currents in oocytes injected with m3 and m2 transcripts. (**a**) Current traces induced by 50 μM ACh application (downward arrows) in three separate oocytes 2 days after injection with m3 transcripts (100 ng/oocyte). The holding potential for all voltage clamped oocytes was −70 mV. The time scale is three times that shown in lower time-to-peak plots. The peak current magnitude is given in nanoAmps (nA). (**b**) Current traces induced in three separate oocytes 2 days after injection with m2 transcripts (100 ng/oocyte). ACh application (50 μM) is indicated by the downward arrows. Upward arrows indicate removal of ACh from the bath. Time scale is the same as that shown in lower time-to-peak plots. The peak current magnitude of each trace is given in nA. (**c**) Plot of peak current versus time-to-peak after ACh application for individual oocytes injected with m3 transcripts. Grey shaded area indicates those oocytes which responded within 20 s of ACh application. The m3 receptor is depicted in the lower right as a seven transmembrane receptor using thick line drawing. (**d**) Same plot as in (c) for individual oocytes injected with m2 transcripts. The m2 receptor is depicted by thin line drawing in the lower right.

Ca^{2+}-activated currents. Several ACh-induced responses from oocytes injected with m2 and m3 transcripts are shown in Figure 2. Based on kinetics and magnitude, each receptor subtype produced a distinctive Cl^- current response when exposed to ACh. Stimulation of oocytes expressing the m3 receptor subtype invariably produced a large and transient inward Cl^- current (Kubo *et al.*, 1986; Fukuda *et al.*, 1987; Bujo *et al.*, 1988). We observed that the rapid onset of the m3-coupled response was largely independent of the magnitude of the stimulated current. For example, m3 dependent peak current magnitudes of 280, 2100 and 10 100 nA occurred within 9, 6 and 4 s of ACh application (Figure 2a). The cumulative results of several hundred oocyte recordings are plotted in Figure 2c and demonstrate that I-Cl activation by the m3 subtype occurred almost exclusively (>98%) within 20 s of ACh application. In contrast, ACh stimulation of oocytes expressing the m2 receptor resulted in much weaker and slower inward current responses (Figure 2b). Additionally, the observed peak current and the time-to-peak current of the m2-coupled responses were much more variable and clearly slower in onset than the m3-coupled responses. As shown in Figure 2b, m2-mediated peak current traces of 100, 210 and 780 nA occurred at 100, 54 and 108 s after ACh application, respectively. Occasionally, a small inward current other than I-Cl was also activated by the m2 subtype, but rarely exceeded 50 nA in magnitude (Figure 2b) (see also Fukuda *et al.*, 1987). The cumulative results of several hundred oocyte recordings indicates that the average time-to-peak of an m2-mediated response occurred 55 s after ACh application. In summary, there are distinct m2 and m3 mediated Ca^{2+}-activated Cl^- currents in oocytes, each coupled to their respective receptors by separate G protein pathways (see below). m3-mediated responses are generally larger in magnitude than those generated through m2 receptors. Additionally, the kinetics of each receptor subtype response, fast for m3 and slow for m2, are constant and independent of the magnitude of a particular response.

The first reciprocal hybrid receptors we examined were Hy9 and Hy10 in which the large cytoplasmic domain joining the fifth and sixth transmembrane segments was precisely exchanged between the m2 and m3 subtypes (Figure 1). We found that the identity of this domain largely determined the type of response evoked by each hybrid receptor (Figure 3). Transfer of the m3 large cytoplasmic domain into the m2 subtype (Hy9) significantly shortened the response time and increased the magnitude of the peak current when compared with the parental m2-coupled response. Notably, 32 of 39 oocytes expressing the Hy9 receptor produced a peak inward current within 20 s of ACh application, remarkably similar to m3-coupled responses (Figure 3a; Table II). Conversely, substitution of the m2 large cytoplasmic domain into the m3 subtype (Hy10) considerably lengthened response times and reduced the magnitude of peak currents when compared with parental m3 responses, effectively producing currents comparable with m2-coupled responses (Figure 3b). Significantly, only four of 30 oocytes expressing the Hy10 receptor produced peak currents in <20 s following ACh application.

Within the mAChR family, protein sequences near the fifth transmembrane segment within the large cytoplasmic domain are conserved between functionally similar subtypes (Peralta *et al.*, 1987b). To examine the importance of this region in G protein activation, we constructed hybrid receptors Hy11 and Hy12 (Figure 1). The Hy11 receptor is comprised of m2 sequences except that the first 21 amino-terminal residues of the third cytoplasmic domain have been exchanged with the corresponding m3 sequences. The Hy11 receptor produced current responses in oocytes virtually indistinguishable from those evoked by Hy9 and m3 receptors; the characteristic time to peak current in 51 of 63 oocytes injected with Hy11 transcripts occurred within 20 s of ACh application (Figure 3c; Table II). Agonist stimulation of the reciprocal Hy12 receptor, an m3 receptor in which the first 21 amino-terminal residues of the third cytoplasmic domain are exchanged for the corresponding m2 sequences, produced a current response similar to the response mediated through Hy10 and m2 receptors (Figure 3d; Table II). Notably, only five of 68 oocytes expressing Hy12 receptors responded within 20 s of ACh addition. From these experiments we concluded that the amino-

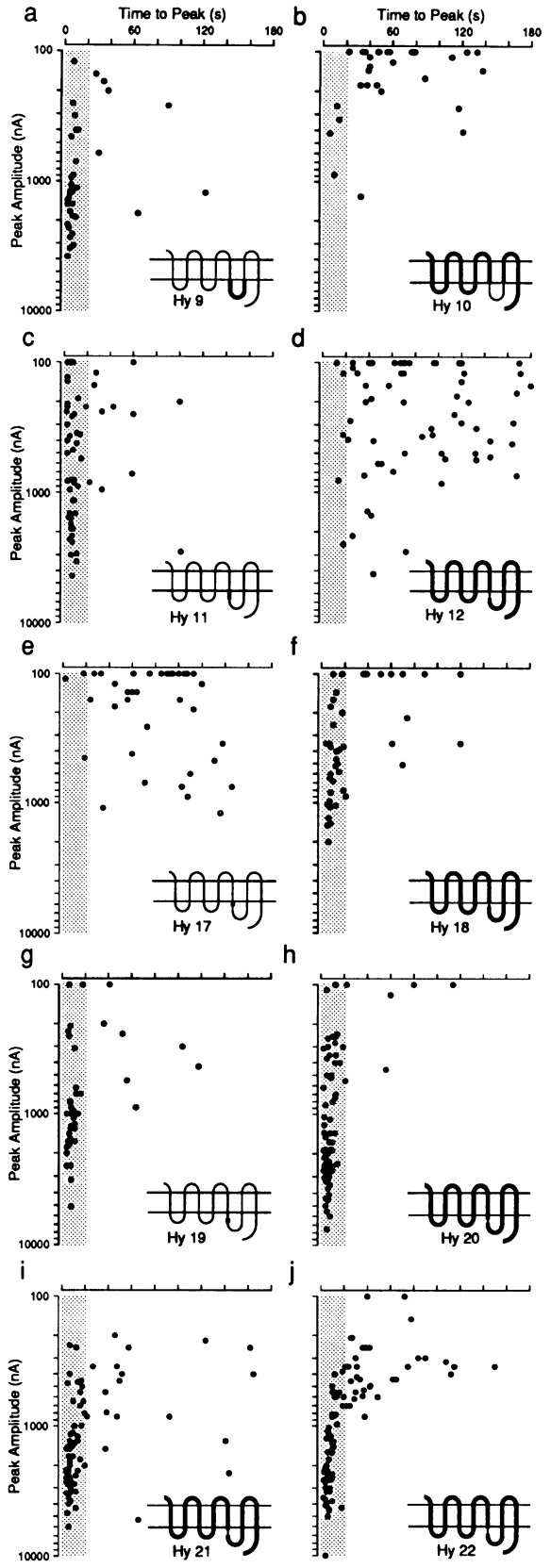

Fig. 3. Peak current versus time-to-peak current plots of individual oocytes injected with m3−m2 hybrid receptor transcripts following ACh application. The particular hybrid receptor examined is indicated in the lower right of each plot. The approximate sequence contributed by m3 and m2 receptors to each hybrid is depicted by thick and thin line drawings, respectively (see Figure 1 and Materials and methods for actual sequences). All experimental conditions are as described in Figure 2. The grey shaded area indicates peak responses which occurred within 20 s after ACh application.

terminal 21 amino acids of the m3 large cytoplasmic domain contained residues critical for conferring the rapid m3-coupled activation of the Ca^{2+} second messenger pathway and suspected that the corresponding residues of the m2 receptor played a similar role in specifying coupling to the delayed Ca^{2+} signaling pathway.

To identify further the critical residues that determine the specificity of G protein coupling by mAChR subtypes, we divided the 21 residue sequence exchanged between Hy11 and Hy12 into amino- and carboxy-terminal segments to construct reciprocal hybrid receptor pairs Hy17 and Hy18, as well as Hy19 and Hy20 (Figure 1). ACh activation of the two hybrid receptors in which the amino-terminal 12 residues were exchanged between the m2 and m3 receptors, Hy17 and Hy18, demonstrated that this region does not change the specificity of G protein coupling from the parental receptors (Figure 3e and f). For oocytes expressing Hy17 receptors, only three of 36 responses occurred within 20 s of ACh application, similar to the delayed and weak responses observed with oocytes expressing parental m2 receptors. In 30 of 43 oocytes expressing Hy18 receptors, the peak ACh-induced response was within 20 s, similar to the parental m3-like responses (Figure 3e and f). These data indicated that the amino-terminal 12 residues of the third cytoplasmic loop could not by themselves convey the G protein coupling properties of either parental receptor. However, exchange of the nine carboxy-terminal residues from the 21 amino acid domain exchanged in Hy11 and Hy12 resulted in substantially different results. Functional analysis of Hy19, an m2 receptor containing only nine amino acids derived from m3, revealed that this hybrid receptor efficiently mediated the rapid kinetic responses characteristic of the m3 subtype. In oocytes expressing Hy19 receptors, 36 of 43 ACh-induced responses occurred within 20 s and the magnitude of the peak current was markedly increased (Figure 3g; Table II). Somewhat paradoxically, the reciprocal Hy20 receptor, in which these same nine amino acids of m3 were exchanged for the corresponding m2 sequence, did not exhibit appreciably different ACh-induced responses from those of the parental m3 receptor itself. In 67 of 72 oocytes expressing Hy20 receptors, the ACh-induced responses were still within 20 s and of greater magnitudes than the m2-like responses (Figure 3h; Table II). These data suggested that the nine carboxy residues transferred into Hy19 were sufficient for specifying m3-like responses, but that other sequences within the m3 receptor were also involved in mediating the m3-like current responses of Hy20. However, the m2-like coupling observed with the Hy12 receptor also suggested that these additional sequences occurred within the amino-terminal 12 residues of the m3 third cytoplasmic domain (Figure 1). To test this possibility, we constructed two additional chimeric receptors, Hy21 and Hy22, in which the nine amino acid segment exchanged in Hy20 was increased to 12 and 17 residues derived from m2, respectively (Figure 1). Interestingly, functional analysis of the Hy21 and Hy22 receptors expressed in oocytes yielded results in which the majority of responses were still m3-like in both kinetics and magnitude. ACh-induced current responses occurred within 20 s in 57 of 78 and 62 of 98 oocytes expressing Hy21 and Hy22 receptors, respectively (Figure 3i and j; Table II). However, both Hy21 and Hy22 receptors exhibited a significant number of ACh-induced oocyte responses with m2-like characteristics, suggesting that these hybrid receptors

Table II. Mean ACh-induced current responses and oocyte expression levels for parental, hybrid and mutant mAChRs

Receptor type	Number[a]	Current (nA/oocyte)	Time-to-peak (s)	B_{max} (fmol/oocyte)
Parental				
m3	454	3101 ± 114	6 ± 1	0.7
m2	304	707 ± 50	55 ± 3	3.8
Hybrid				
Hy9	39	1233 ± 153	16 ± 4	1.3
Hy10	30	217 ± 48	59 ± 7	0.4
Hy11	63	857 ± 295	15 ± 6	0.4
Hy12	68	499 ± 318	75 ± 13	0.3
Hy17	36	310 ± 58	78 ± 6	1.8
Hy18	43	512 ± 71	27 ± 5	0.3
Hy19	43	1129 ± 278	18 ± 8	5.1
Hy20	72	2076 ± 457	9 ± 3	1.3
Hy21	78	1668 ± 324	26 ± 13	0.4
Hy22	98	1543 ± 386	25 ± 17	1.2
Deletion mutants				
m3ΔYKETEKRTKE	26	243 ± 35	97 ± 9	0.1
m3ΔKEKK	23	2993 ± 301	4 ± 1	1.3
m3ΔKRKRMSLV	25	813 ± 235	5 ± 1	<0.05

[a]Number in this column refers only to the number of oocytes electrically recorded from. For individual binding assays, 20–70 oocytes were used except for the parental B_{max} where the mean of all experiments for m2 and m3 receptor expression is given.

may be coupling to both the m2 and m3 G protein pathways. Therefore, these data suggest that most, or all of the 21 amino acids exchanged in Hy12 are necessary to specify the m2-coupled pathway of Ca^{2+} release.

Deletion mutants identify essential and non-essential sequences within the G protein coupling domain of the m3 subtype

The functional analysis of hybrid mAChRs demonstrated that the 21 amino acids adjacent to the fifth transmembrane segment of the m2 and m3 subtypes are crucial in determining the specificity of G protein activation by a particular receptor subtype. To investigate the importance of this region in G protein activation further, a deletion mutant of the m3 subtype (m3ΔYKETEKRTKE) was constructed in which 10 residues near the amino-terminal end of the third cytoplasmic domain were eliminated (Figure 1). Deletion of these sequences completely altered the kinetics and magnitude of the responses compared with the parental m3 receptor. Only one of 26 oocytes expressing m3ΔYKETEKRTKE receptors responded within 20 s of ACh application. Moreover, the magnitudes of the peak currents were substantially lower than those evoked by the m3 receptor (Figure 4c). Therefore, although hybrid receptor analysis suggested that this region of the third cytoplasmic domain does not play a critical role in determining the G protein specificity of receptor subtypes, deletion mutagenesis demonstrated that this region does perform a critical function in some aspect of the G protein activation process.

As noted above, functionally similar mAChR subtypes also share a limited amount of sequence similarity in regions of the third cytoplasmic domain adjacent to the sixth transmembrane segment. Hybrid receptor analysis demonstrated that critical amino acids controlling G protein specificity reside near the fifth transmembrane segment; therefore, we wished to determine through deletion mutagenesis of the m3 subtype whether conserved sequences near the sixth transmembrane segment may be required for some distinct aspect of G protein activation. Deletion

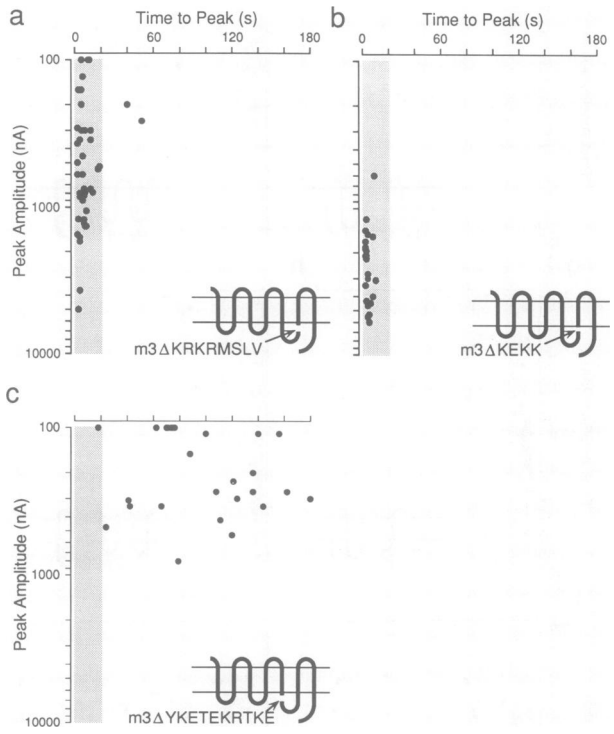

Fig. 4. Peak current versus time-to-peak current plots of individual oocytes injected with m3 deletion mutant transcripts following ACh application. The parental m3 receptors are depicted by thick line drawings. The specific sequence deleted is listed; the approximate location within the m3 receptor is indicated with an arrow and shown as an interruption in the thick line drawings. Conditions are as described in Figures 2 and 3.

mutant m3ΔKRKRMSLV lacks eight conserved basic or hydrophobic amino acids while m3ΔKEKK lacks four charged residues which are conserved between all mAChR subtypes. Functional expression of these deletion mutant receptors in oocytes resulted in rapid kinetic responses with peak currents that occurred within 20 s after ACh application

(Figure 4a and b). The magnitudes of the peak currents were also quite similar to the parental m3-like responses, although the peak currents evoked by the m3ΔKRKRMSLV receptor were somewhat reduced compared with wild-type m3 responses. Thus, conserved cytoplasmic sequences near the sixth transmembrane domain are not essential for the rapid m3-like activation of the G protein pathway leading to intracellular calcium release.

Sensitivity to pertussis toxin reveals the reciprocal nature of hybrid receptor responses

The experiments described above identified important residues in the third cytoplasmic domain adjacent to the fifth transmembrane segment which are critically involved in determining whether ACh evokes either an m2-like or m3-like response via a particular mAChR subtype. We interpreted a change in the kinetics and magnitude of receptor-coupled responses as indicative of a change in the identity of the G proteins which couple the receptor to the Ca^{2+} second messenger pathway. Multiple types of G proteins appear to couple receptors to the activation of PLC since receptor-mediated PI hydrolysis is blocked by PTX in some, but not all cell types (Cockcroft, 1987; Ashkenazi *et al.*, 1989). Previous studies with transfected cell lines established that the activation of PI hydrolysis by the m2 and m3 subtypes can be distinguished on the basis of differential sensitivity to PTX; the limited response observed through activation of the m2 receptor can be completely blocked by PTX, while the large PI response mediated through the m3 subtype is largely resistant to the toxin (Ashkenazi *et al.*, 1989; Lechleiter *et al.*, 1989). Therefore, we wished to determine if PTX could be used to distinguish between the coupling properties of normal and hybrid receptors within the oocyte expression system.

Initial experiments established that the large inward transient Cl^- current (D1) mediated by the m3 receptor subtype was largely resistant to PTX treatment, while the delayed and fluctuating Cl^- current (F) activated by m2 receptors was almost entirely abolished by the toxin (Figure 5). In parallel experiments, we tested the sensitivity of ACh-induced responses with oocytes expressing either Hy11 or Hy12 receptors. As predicted by the kinetics and magnitudes of the ACh-induced current responses, we found that the source of the 21 amino acid cytoplasmic domain determined the sensitivity of the oocyte responses to PTX, rather than the unsubstituted regions of the parental receptor (Figure 5). Agonist stimulation of Hy11 receptors produced rapid m3-like currents which were largely resistant to the toxin. In contrast, the delayed, m2-like currents mediated by Hy12 receptors were markedly inhibited by PTX (Figure 5). These data provide direct evidence that the G protein specificity of a receptor can be changed in a reciprocal manner between m2 and m3 subtypes by exchanging the amino-terminal residues near the fifth transmembrane segment of the third cytoplasmic domain.

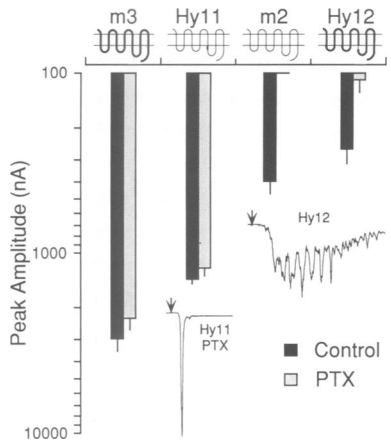

Fig. 5. Pertussis toxin (PTX) sensitivity of the m3- and m2-coupled responses in oocytes are reversed for hybrid receptors Hy11 and Hy12. The mean ACh-induced peak currents in nA (±SEM) in the absence of PTX were 3030 ± 417 (n = 25) for m3, 1387 ± 172 (n = 18) for Hy11, 397 ± 69 (n = 25) for m2 and 260 ± 56 (n = 30) for Hy12 receptors. In paired experiments, half of the injected oocytes were treated with PTX (2 μg/ml, 20 h). The mean PTX treated peak currents were 2325 ± 470 (n = 28) for m3, 1225 ± 201 (n = 18) for Hy11, 59 ± 15 (n = 25) for m2 and 109 ± 18 (n = 29) for Hy12 receptors. Insets show a Hy11 PTX treated oocyte with a peak current response of 2750 nA and a Hy12 receptor response (no PTX) with a peak current of 1400 nA. Arrow indicates time of ACh application. Time-to-peak current for the insets was 10 s for Hy11 PTX treated oocyte and 39 s for the control Hy12 response.

Discussion

By analyzing the ability of hybrid mAChRs to activate differentially Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes, we have identified a region of 21 amino acids within the third cytoplasmic domain which plays a critical role in determining the specificity of G protein activation by receptor subtypes. In fact, as few as nine amino acids from the 241 residue third cytoplasmic domain of the m3 subtype appeared largely sufficient to convert the G protein-coupled responses of a receptor from m2- to m3-like characteristics. These data support and dramatically extend the work of other investigators which identified the third cytoplasmic domain as a critical element in determining the specificity of receptor–G protein interaction (Kobilka *et al.*, 1988; Kubo *et al.*, 1988; Dixon *et al.*, 1989; Shapiro and Nathanson, 1989; Wess *et al.*, 1990).

The G protein-coupled responses of normal, hybrid and deletion mutant receptors were evaluated by two major criteria: the kinetics of I-Cl activation and the magnitude of peak I-Cl currents. Those receptors with m3-like responses displayed rapid and transient ACh-induced D1 currents, while receptors with m2-like responses consistently activated F currents which were delayed in onset, oscillatory and of lower magnitude. Moreover, the truly reciprocal nature of the exchange in G protein specificity displayed by hybrid receptors containing the critical 21 amino acid domain (Hy11 and Hy12) was established by determining the PTX sensitivity of the receptor-mediated responses. Specifically, the rapid activation of D1 currents mediated by m3 and Hy11 (m2 containing 21 amino acids from m3) was insensitive to PTX, while the smaller F currents predominantly activated by m2 and Hy12 (m3 containing 21 amino acids from m2) were blocked by PTX (see Figure 5). These experiments provide the first evidence that m2 and m3 receptor subtypes utilize the same region of the third cytoplasmic domain to control the specificity of interaction with PTX sensitive or insensitive G proteins, respectively. However, the non-reciprocal nature of Hy19 and Hy20 responses suggests that other regions may be required to specify fully coupling to the m2 pathway. Furthermore, these data demonstrate

Table III. Alignment of amino-terminal residues of the third cytoplasmic loop of PI-coupled receptors

m3-like receptors	
m1	R I Y R E T E N R A R – E L A A L Q G S E T P G K G G
m3	R I Y K E T E K R T K – E L A G L Q A S G T E A E T E
m5	R I Y R E T E K R T K – D L A D L Q G S D S V T K A E
5HT1C	T I Y V L – – – R – R Q T L M L L R G H T E E E L A N
5HT2B	T I K S L Q – K E A – – T L C V S D L S T R A K L A S
α1 adrenergic	R V Y I V A – K R T T K N L E A G V M K E M S N S K E
m2-like receptors	
m2	H I S R A S K S R I K K D K K E P V A N Q D P V S P S
m4	H I S L A S R S R V H K H R P E G P K E K K A K T L A
Wasp venom mastoparan	L I K K A L A A L A K L N I

distinct G protein pathways which appear to activate PLC and intracellular Ca^{2+} release differentially. Since mAChR subtypes also regulate adenylyl cyclase activity through G proteins, these hybrid receptors will also be tested for their ability to regulate intracellular cAMP levels (Peralta *et al.*, 1988).

The regulation of G protein activation has now been examined for several subtypes of adrenergic and muscarinic receptors. Recently, experiments in which cytoplasmic sequences of the β2 adrenergic receptor were substituted for corresponding regions of the m1 mAChR resulted in a muscarinic agonist responsive receptor coupled to the stimulation of adenylyl cyclase (Wong *et al.*, 1990). Interestingly, one such hybrid receptor was able to activate adenylyl cyclase upon acquiring β2 adrenergic receptor sequences proximal to the fifth transmembrane domain, yet the character of muscarinic coupling to PI hydrolysis was unaffected (Wong *et al.*, 1990). These findings are not necessarily in conflict with those reported here since the assays of receptor function were quite different. In particular, differences in kinetic responses of receptors are not as readily apparent in biochemical assays such as inositol phosphate measurements, when compared with the higher time resolution attainable through an electrophysiological assay. Other recent studies have identified key regions within the third cytoplasmic domain of the α1 adrenergic receptor which are important for efficient coupling to PI turnover (Cottechia *et al.*, 1990). Notably, these studies revealed that conservative substitutions near the carboxy-terminal end of the third cytoplasmic domain could enhance adrenergic receptor stimulation of PI hydrolysis (Cottechia *et al.*, 1990). The mAChR family appears to differ from the adrenergic receptor family in this regard as deletions of conserved sequences near the carboxy-terminal end of the third cytoplasmic domain had little effect on m3-mediated stimulation of Ca^{2+}-activated Cl^- currents (Figure 4a and b). Since G proteins mediating the PTX insensitive pathway of PLC activation have yet to be identified, it is unknown whether m3 mAChRs and α1 adrenergic receptors interact with the same G protein.

Deletion mutagenesis within the amino-terminal portion of the 21 residue G protein selectivity domain drastically altered the kinetics and magnitude of the m3-coupled responses, indicating that these sequences are required for G protein activation. However, analysis of hybrid receptors in which the amino-terminal 12 residues of this region were exchanged between the m2 and m3 receptors (Hy17 and Hy18) established that these sequences, while important for some aspect of G protein activation, do not confer specificity to this interaction. Rather, the smallest sequence which conferred m3-like responses to the m2 subtype was the nine amino acid sequence LAGLQASGT (Table III). Using various peptide structure algorithms we compared regions containing this sequence within the mAChR family and with other PLC-coupled receptors such as the α1 adrenergic and the 5HT2B and 5HT1C serotonin receptors (Table III) (Devereux *et al.*, 1984). This comparison identified a region of potentially amphipathic, α-helical structure followed by a somewhat hydrophobic sequence analogous to the selectivity domain of the m3 receptor identified in this study (Table III). Based on the ability of the wasp toxin mastoparan, a putative amphipathic, α-helical peptide, to activate the GTPase activity of purified G proteins, others have proposed that such a receptor structure is critical for G protein activation (Higashijima *et al.*, 1988). Preliminary testing of this notion, via an insertion mutation which should rotate the putative helix (an alanine insertion between residues Glu259 and Lys260 of m3), resulted in reduction of the magnitude, but not the rapid kinetics of an m3 response (unpublished observations).

The comparisons described above suggest that functionally similar receptors may utilize domains of conserved secondary structure and ionic character, but limited sequence identity, to interact with the same or similar G proteins. Conservation of helical secondary structure and amphipathic charge distribution is likely to be an important aspect of some protein surfaces involved in the association of transcription factors (Giniger and Ptashne, 1987). However, assignment of a single attribute to receptor domains involved in controlling the specificity of G protein activation remains speculative. For example, while m3-like responses could be transferred to an m2 receptor by the exchange of only nine amino acids (Hy19), 21 residues derived from m2 were minimally required to transfer m2-like responses to the m3 subtype (Hy12). This suggests that a region of different hydrophobicity and perhaps flexibility is required to confer m2 specific G protein coupling. In addition, the ability of Hy20, Hy21 and Hy22 to still confer m3-like responses suggests that other m3 sequences may be important in controlling G protein interactions (Wong *et al.*, 1990). The role of the second cytoplasmic domain and carboxy terminus of mAChR subtypes in G protein activation is currently under investigation.

In addition to providing a sensitive assay for the G

protein coupling properties of mAChR subtypes, electrophysiological measurements of Ca^{2+}-activated Cl^- currents have revealed interesting differences in the pathways by which subtypes of the same receptor family can activate intracellular Ca^{2+} release. Based on the profiles of the Ca^{2+}-activated Cl^- currents, agonist stimulation of the m3 subtype must induce a rapid release of intracellular Ca^{2+} which returns to basal levels nearly as rapidly as the initial ascent. In contrast, ACh stimulation of the m2 subtype probably produces a slowly building, generally oscillatory increase in intracellular Ca^{2+}. Any return to basal levels of Ca^{2+}, in the continued presence of ACh, is slow and on many occasions continues to oscillate above basal levels for the duration of an experiment (>3 min). Furthermore, the peak magnitudes of the m2-like Ca^{2+} releases are generally smaller in amplitude when compared with m3-like responses. Presumably, both the m2 and m3-coupled Ca^{2+} release pathways are initiated through the production of IP_3, yet the cell is able to utilize this second messenger molecule to evoke very distinct forms of Ca^{2+} responses. Different receptors may thus encode their specific cellular signals through the activation of distinct G protein pathways which ultimately allow modulation of both the amplitude and frequency of Ca^{2+} release.

Materials and methods

Construction of hybrid and deletion mutant receptors

Isolation of plasmids containing the entire coding sequence of the m2 and m3 subtypes was described previously (Peralta *et al.*, 1987b). To construct hybrid receptor genes containing fusions of coding sequences at any desired location, the polymerase chain reaction (PCR) was used to amplify selectively DNA fragments encoding portions of muscarinic receptor proteins from plasmids containing the normal human m2 and m3 genes. In each case the 5′ portion of the hybrid gene was amplified with a forward primer encoding a unique restriction site (*Eco*RI) and the amino-terminal portion of the hybrid mAChR. The reverse primer for this fragment encoded the seven amino acids at the site of the fusion. The 3′ portion of each hybrid receptor gene was amplified with a forward primer encoding the amino-terminal seven amino acids on the other side of the fusion site, while the reverse primer for this fragment encoded a second unique restriction site (*Xba*I) and the seven residues at the carboxy terminus of the hybrid receptor protein. Each fragment was amplified from 10–50 ng of plasmid DNA by 25 cycles consisting of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C as described by the manufacturer (Cetus). The amplified fragments were treated with T4 DNA polymerase in the presence of all four deoxyribonucleoside triphosphates to create blunt ends, digested with *Eco*RI or *Xba*I, and gel purified. The amino-terminal portion of each hybrid receptor was thus encoded within a fragment containing an *Eco*RI overhang at the 5′ end and a blunt 3′ end. The carboxy-terminal portion of each hybrid was encoded by a fragment containing a blunt 5′ end and a 3′ end containing an *Xba*I overhang. Fragments encoding the 5′ and 3′ portions of each hybrid receptor were ligated with *Eco*RI and *Xba*I digested pGEM3 (Promega). Due to the orientation specific nature of this three fragment ligation, a hybrid mAChR gene is obtained containing unique *Eco*RI and *Xba*I sites at the 5′ and 3′ ends of the gene, respectively, while fusion of the coding sequences occurs by blunt end ligation. The DNA sequences of the resulting hybrid receptor genes were confirmed as described by Tabor and Richardson (1987). The error rate of Taq DNA polymerase was found to be <1 in 10 000 bases under the conditions described by the manufacturer (Cetus). The hybrid receptor genes were also inserted into a mammalian cell expression vector under the transcriptional regulation of the cytomegalovirus (CMV) early promoter.

The amino acid sequences described here are from the human mAChRs described previously (Peralta *et al.*, 1987b). The following residues were exchanged between the m2 and m3 subtypes in hybrid receptors: Hy9 contains Arg253–Gln491 of m3 substituted for His208–Arg387 of m2; Hy10 contains His208–Arg387 of m2 substituted for Arg253–Gln491 of m3; Hy11 contains Arg253–Thr273 of m3 substituted for His208–Gln228 of m2; Hy12 contains His208–Gln228 of m2 substituted for Arg253–Thr273 of m3; Hy17 contains Arg253–Glu264 of m3 substituted for His208–Lys219 of m2; Hy18 contains His208–Lys219 of m2 substituted for Arg253–Thr273 of m3; Hy19 contains Leu265–Thr273 of m3 substituted for Asp220–Gln228 of m2; Hy20 contains Asp220–Gln228 of m2 substituted for Leu265–Thr273 of m3; Hy21 contains Ile217–Gln228 of m2 substituted for Thr262–Thr273 of m3; Hy22 contains Ala212–Gln228 of m2 substituted for Glu257–Thr273 of m3.

Deletion mutant derivatives of the m3 mAChR subtype and hybrid receptors Hy19–22 were constructed by standard oligonucleotide-directed mutagenesis of single stranded DNA templates as described (Kunkel *et al.*, 1987). The sequence of each deletion mutant was confirmed by DNA sequence analysis (Tabor and Richardson, 1987).

Cell culture and transfections

Plasmids encoding each hybrid or deletion mutant mAChR under the transcriptional control of the CMV early promoter were cotransfected with plasmid pRSVNeo by the calcium phosphate method into human embryonic kidney cells (ATCC no. CRL1573) as described (Peralta *et al.*, 1988). Transfected cells were selected for resistance to 500 μg/ml G418 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/ml penicillin and streptomycin. G418 resistant cells were massed (with the exception of Hy11 transfected cells which were obtained by single cell cloning) and the levels of mAChRs were determined by saturation binding of the non-selective muscarinic antagonist [^{3}H] methyl scopolamine (NMS) to intact cells as described (Peralta *et al.*, 1987a). Data in Table I were derived from three or more experiments. Non-transfected cells expressed <400 NMS binding sites per cell. Agonist binding was assayed by competition displacement with [^{3}H]NMS and the acetylcholine analog carbachol as described (Peralta *et al.*, 1987b). Antagonist and agonist binding date were analyzed using the LIGAND program (Munson and Rodbard, 1980).

In vitro transcriptions and oocyte methods

In vitro capped transcripts were synthesized from linearized plasmid template DNA with SP6 RNA polymerase using standard protocols (Melton *et al.*, 1984). *In vitro* transcripts were resuspended in water at a final concentration of ~2 μg/μl.

Adult female *Xenopus laevis* toads were obtained from Nasco (Fort Atkinson, WI, USA) and Carolina Biological (Burlington, NC). Ovarian lobes were removed under sterile conditions from anesthetized animals followed by immediate suturing of the incisions. Animals were allowed to recover in isolation before being returned to the aquaria; this allowed individual toads to be used again following healing of surgical incisions.

Stage V and VI oocytes were mechanically defolliculated with fine forceps within 12 h after removal from the animal. Defolliculated oocytes were kept in L-15 supplemented medium at 19°C and injected with RNA transcripts 24 h after surgical removal. Injection electrodes were broken to tip diameters between 14 and 16 μm and baked at 250°C. SP6 transcripts (100 ng/oocyte) were injected in a 50 nl bolus using a 10 μl Drummond micropipet. Injected oocytes were stored in L-15 supplemented media at 19°C for 2 days with one change of media prior to recording.

Electrophysiology

Two electrode voltage clamp (Turbo TEC 01C, mpi Instruments, Germany; Axoclamp 2A, Axon Instruments, Foster City, CA) was used to measure the ACh-induced currents from individual oocytes. Recordings were made in Barth's solution at room temperature. Current and potential recording electrodes were filled with 2 M KCl and pulled to resistances of 0.5 MΩ and 2 MΩ, respectively. All oocytes were clamped at −70 mV. Current records were stored on a video cassette recorder equipped with a digital pulse code modulator (SONY, 501ES).

For each experiment, 300–480 oocytes were defolliculated and divided into groups of 60. Three of the groups were always injected separately with m2, m3 and sterile dH_2O. This allowed us to continuously monitor the ability of the oocytes to express parental m2 and m3 receptors as well as to detect the infrequent occurrence of an endogenous muscarinic current response. ACh-induced current responses from oocytes injected with hybrid or deletion mutant transcripts were not included in our data pool unless their peak current was greater than the mean control current (from dH_2O injected oocytes) plus one standard error of the mean. Over the course of 10 months, the mean control current (dH_2O injected alone) averaged <30 nA.

Nomenclature

In this manuscript we have adopted the nomenclature for muscarinic receptor subtypes as determined at the Fourth International Symposium on Subtypes of Muscarinic Receptors (Weisbaden, FRG, 1989). Therefore, the human m3 and m4 receptors described in this manuscript correspond to the HM4 and HM3 receptors, respectively, described in previous work (Peralta *et al.*, 1987b, 1988; Ashkenazi *et al.*, 1989).

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