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Charged amino acids required for signal transduction by the m3 muscarinic acetylcholine receptor

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The five muscarinic acetylcholine receptor (mAChR) subtypes, termed m1-m5, transduce agonist signals across the plasma membrane by activating guanine nucleotide binding (G) proteins. The large cytoplasmic domain joining the fifth and sixth transmembrane segments of mAChRs plays a critical role in controlling the specificity of G protein coupling. In this study, we determined which sequences within this domain are required for activation of signaling by the m3 mAChR. By measuring the ability of normal and mutant m3 mAChRs to couple to the G protein pathway leading to activation of phospholipase C and Ca^{2+}-dependent chloride currents in RNA-injected *Xenopus* oocytes, we found that two clusters of charged residues near the fifth and sixth transmembrane segments were required for normal signaling; furthermore, the position of these sequences was critical for their function. Finally, analysis of deletion mutant m3 mAChRs confirmed the importance of these sequences; receptors containing as few as 22 out of 239 amino acids of the cytoplasmic domain were fully active in signaling if they included the critical charged residues. Sequence comparisons suggest that similar charged sequences may be required for signal transduction by many G protein-coupled receptors.

Key words: acetylcholine/G proteins/receptors/signaling/transduction

Introduction

Muscarinic acetylcholine receptors (mAChRs) belong to a large family of seven transmembrane receptors which evoke distinct intracellular responses through the activation of heterotrimeric, guanine nucleotide binding (G) proteins. Agonist-bound mAChRs activate heterotrimeric G proteins by promoting guanine nucleotide exchange; the GTP-bound α subunits or free $\beta\gamma$ subunits then regulate specific effector enzymes or ion channels (Birnbaumer, 1992; Kaziro *et al.*, 1991). The five mAChRs can be divided into two classes based on structural and functional similarities: the structurally related m1, m3 and m5 receptor subtypes potently activate the effector enzyme phospholipase C (PLC), while the related m2 and m4 receptors weakly activate PLC and efficiently inhibit adenylyl cyclase (Peralta *et al.*, 1988; Ashkenazi *et al.*, 1989). Activation of PLC leads to the generation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol which in turn mediate the release of intracellular calcium and stimulation of protein kinase C, respectively (Berridge, 1993). The G proteins that mediate signaling by the two functional classes of mAChRs can be differentiated on the basis of their sensitivity to pertussis toxin (PTX), a bacterial toxin which ADP-ribosylates certain G_α subunits, thus blocking their interaction with receptors (Kaziro *et al.*, 1991). Activation of PLC by the m1, m3 and m5 mAChRs is insensitive to PTX; in contrast, inhibition of adenylyl cyclase and stimulation of PLC via the m2 and m4 mAChRs is mediated by PTX-sensitive G proteins (Ashkenazi *et al.*, 1989; Lechleiter *et al.*, 1990; Dell'Acqua *et al.*, 1993).

Studies of chimeric receptors have demonstrated the importance of the third cytoplasmic domain joining the fifth and sixth transmembrane segments in controlling the specificity of G protein coupling (Kubo *et al.*, 1988; Cotecchia *et al.*, 1990; Lechleiter *et al.*, 1990; Wong *et al.*, 1990; England *et al.*, 1991). Exchange of the entire domain between functionally distinct $\alpha 2$ and $\beta 2$ adrenergic receptors converts the G protein selectivity of the resulting hybrid receptors (Kobilka *et al.*, 1988). Moreover, analysis of hybrid receptors derived from the m2 and m3 mAChR subtypes demonstrates that within the third cytoplasmic domain, amino acids near the fifth transmembrane segment play a particularly important role in determining G protein specificity (Lechleiter *et al.*, 1990; Wess *et al.*, 1990). For example, a derivative of the m2 mAChR in which the first 21 residues of the large third cytoplasmic domain are derived from the m3 mAChR strongly activates PLC-dependent responses through a PTX-resistant G protein pathway, while the reciprocal m3-based receptor containing the corresponding region of the m2 mAChR weakly stimulates PLC via a PTX-sensitive G protein (Lechleiter *et al.*, 1990). Therefore, functional analysis of chimeric receptors has shown that specific amino acids within the third cytoplasmic domain play a critical role in determining which G proteins may interact with a given receptor. However, previous studies do not reveal whether these sequences, or other regions within the third cytoplasmic domain, are required for the activation of G protein signaling.

Synthetic and naturally occurring peptides have been employed to identify sequences capable of activating heterotrimeric G proteins. Several *in vitro* studies have shown that relatively short peptides of 19 residues or less are capable of stimulating the GTPase activity of certain G proteins. For example, mastoparan, a peptide component of wasp venom, stimulates the GTPase activity of several G protein α subunits including $G_{\alpha o}$, $G_{\alpha i}$ and $G_{\alpha s}$ (Higashijima *et al.*, 1990). More recently, synthetic peptides corresponding to sequences found within the third cytoplasmic domain of several receptors, such as the $\alpha 2$-adrenergic receptor and m4 mAChR, have been shown to stimulate the GTPase activity of several G protein α subunits (Okamoto and Nishimoto, 1992). Each of the G protein activating peptides contains several basic amino acids which may form the charged surface of an amphipathic α helix. It has been suggested from these studies that GTPase activating peptides

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mimic a receptor domain involved in G protein activation; however, the sequence requirements for G protein activation have not been systematically tested within the context of a G protein-coupled receptor expressed *in vivo*.

In this study, we determined which residues within the 239 amino acid third cytoplasmic domain of the m3 mAChR are required for normal activation of the G protein pathway leading to stimulation of PLC and intracellular Ca^{2+} release. Our experiments demonstrated that a small number of charged residues at the amino- and carboxy-termini of the third intracellular domain are critical for the activation of G protein signaling; furthermore, the correct positioning of these sequences was essential for their activity. Sequence comparisons revealed that the important residues identified by these experiments are conserved within substantially divergent G protein-coupled receptors. We suggest that a limited number of charged amino acids within the third cytoplasmic domain may play an important role in G protein signaling by many types of neurotransmitter and peptide hormone receptors.

Results

Substitution mutations in the m3 receptor identify charged sequences required for G protein signaling

Previous analysis of hybrid receptors derived from the functionally distinct m2 and m3 mAChRs demonstrated that a 21 amino acid sequence immediately adjacent to the fifth transmembrane segment plays a critical role in determining the specificity of G protein coupling (Lechleiter *et al.*, 1990). Eleven of the first 12 residues within this sequence are charged and polar amino acids (Figure 1). Interestingly, reciprocal exchange of these 12 residues between the m2 and m3 receptors does not alter G protein specificity, although a deletion mutation removing all the charged and polar sequences severely impairs G protein activation by the m3 receptor (Lechleiter *et al.*, 1990). However, it is not clear from these studies whether the charged and polar sequences play a role in G protein activation or if deletion of these residues results in the improper positioning of an activating region located elsewhere in the third cytoplasmic domain.

To determine whether the charged or polar residues at the amino-terminal end of the third cytoplasmic domain are required for G protein signaling, we constructed two substitution mutants (m3-A and m3-B) in which five consecutive uncharged residues were substituted within this region of the m3 mAChR (Figure 1). In this study, receptor-stimulated G protein signaling was assayed by recording Ca^{2+}-activated Cl^- currents (I-Cl) from RNA-injected *Xenopus* oocytes expressing wild type or mutant m3 mAChRs. Previous studies have shown that I-Cl responses directly reflect the magnitude of intracellular Ca^{2+} release resulting from mAChR-stimulated PLC activity and IP3 production (Lechleiter *et al.*, 1991). Therefore, this method provided a sensitive *in vivo* assay for the activation of G protein-dependent signaling since we were able to measure both the kinetics and magnitude of I-Cl currents following addition of the muscarinic agonist carbachol (Lechleiter *et al.*, 1990). As shown in Figure 2, agonist stimulation of the wild type m3 mAChR caused a large, rapid and transient activation of I-Cl in RNA-injected oocytes, while carbachol failed to evoke any responses in water-injected control oocytes. Typically, m3 mAChR-induced I-Cl responses were

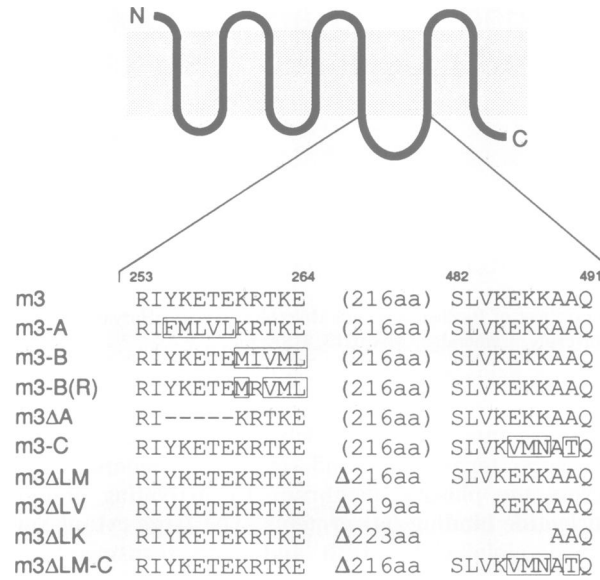

Fig. 1. Structure of wild type, substitution and deletion mutant m3 mAChRs. R_{253} and Q_{491} denote the putative amino- and carboxy-terminal residues of the third cytoplasmic domain, respectively. Regions containing substitution mutations are boxed; deleted residues in m3ΔA are indicated by dashes. The 216 amino acids (216aa) from residues 265–481 are contained in certain receptors, but not shown in this diagram. The minimal receptors are designated according to the boundaries of each deletion mutation; for example, residues $L_{265}-M_{481}$ are deleted in m3ΔLM. The number of amino acids (aa) deleted within minimal receptors is indicated (Δ).

detected within 2 s after carbachol addition, rapidly reached their peak amplitude and quickly declined to baseline. Recordings from oocytes expressing the m3-A receptor demonstrated that substitution of the first five charged or polar residues in the amino-terminal region of the third cytoplasmic domain did not alter the rapid onset or magnitude of I-Cl responses (Figure 2 and Table I). In contrast, substitution of the subsequent five residues severely impaired signaling by the m3-B receptor (Figure 2 and Table I). Currents evoked from m3-B-expressing oocytes were significantly delayed in onset and reduced in peak amplitude compared with responses generated by the parental m3 mAChR; in addition, m3-B currents did not generally display the sharp decay to baseline typical of m3 responses.

Antagonist binding was performed on plasma membranes purified from oocytes expressing the various mutant mAChRs to confirm that any observed decreases in the magnitude or onset time of I-Cl responses were not a consequence of lower receptor expression levels. As shown in Table I, the expression levels of mutant receptors were generally similar to that determined for the wild type m3 mAChR. To confirm that reductions in responses were not due to failure of the receptor to adequately bind carbachol, agonist displacement studies were conducted on membranes derived from stable transfected 293 cells expressing each mutant receptor. As shown in Table I, the carbachol binding constant (K_D) determined for each of the mutant receptors was similar to the wild type m3 receptor and well below the dose (100 μM) used to evoke I-Cl responses. Therefore, these ligand binding studies strongly suggest that deficiencies in the functional responses generated by the m3-B receptor (and other mutant receptors described below) result from a decrease in the ability of the receptor to activate the G protein pathway leading to PLC and I-Cl activation.

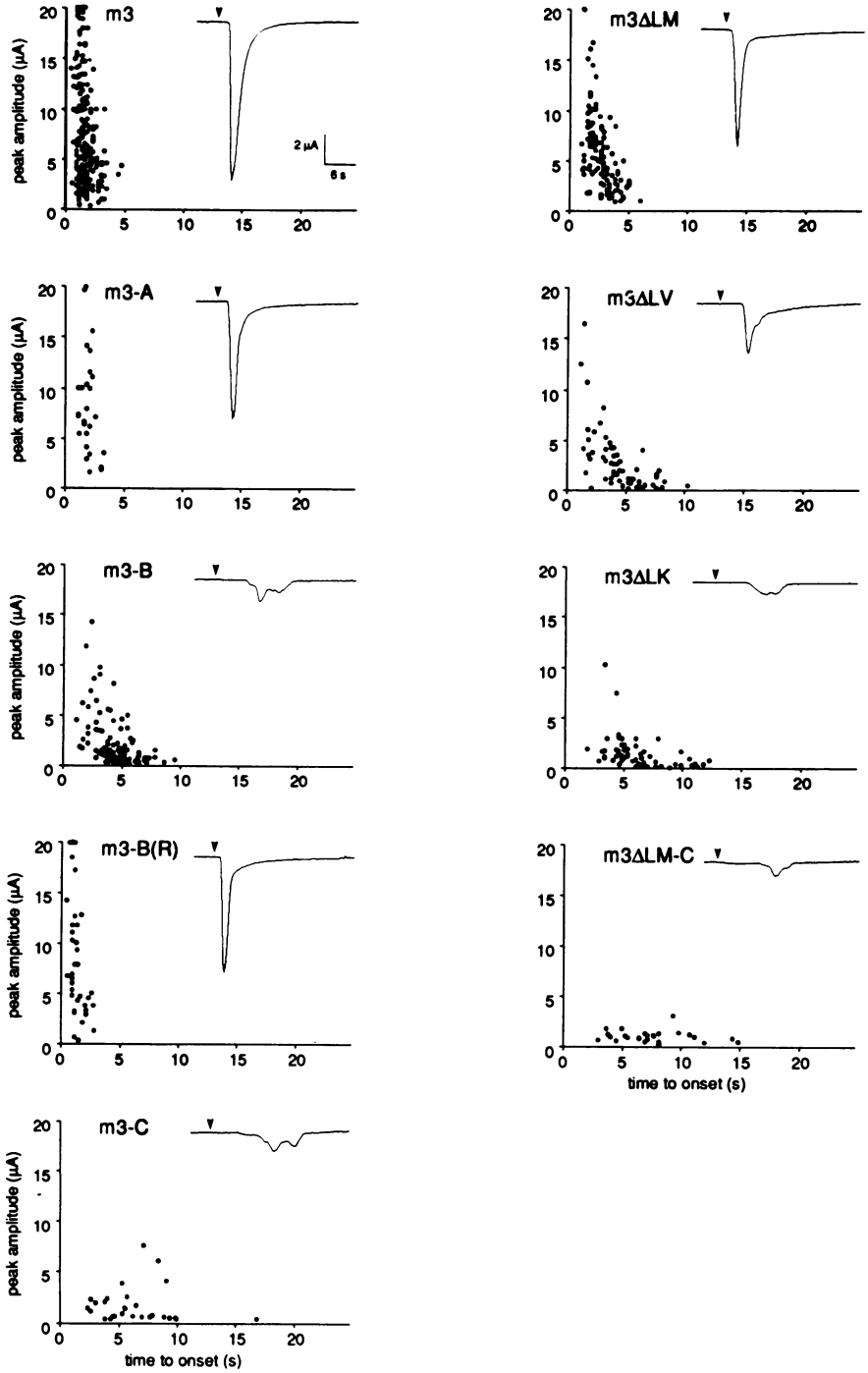

Fig. 2. Carbachol-induced currents in oocytes expressing normal or mutant m3 mAChRs. Ca^{2+}-activated Cl^- currents were evoked from individual oocytes (●) by application of 100 μM carbachol (downward arrow) to oocytes clamped at a holding potential of −70 mV. The peak current amplitude is given in microAmps (μA) and graphed versus the time to current onset in seconds. A representative current trace is shown for each receptor; note that the time and current scale for individual traces is shown in the upper left m3 panel and differs from that used for the graphed data.

The inability of the m3-B receptor to evoke normal I-Cl responses demonstrated that the charged sequence $K_{260}RTKE_{264}$ is important for activation of G protein signaling. To determine more precisely which residues are essential for normal m3 mAChR responses, we reintroduced individual charged residues into the m3-B receptor background (Figure 1 and Table I). Although a derivative of the m3-B receptor containing the lysine at residue 260 evoked stronger carbachol-induced I-Cl responses than the parental m3-B receptor (data not shown), reintroduction of the arginine at residue 261 conferred wild type I-Cl responses on the mutant receptor termed m3-B(R) (Figure 2 and Table I). In addition, to determine whether G protein activation simply requires the charged sequences identified by the m3-B substitution mutation or whether these residues must occupy a specific location within the amino-terminal end of the cytoplasmic domain, we also constructed a mutant receptor, termed m3ΔA, in which the adjacent residues 255–259 were deleted (Figure 1). The m3ΔA mutant displayed no detectable responses to carbachol, although it

Table I. Average responses and receptor expression levels in oocytes expressing wild type and mutant m3 mAChRs

Receptor	Number of oocytes[a]	Time to onset (s)	Peak current (μA)	fmol/100 μg protein
m3	236	1.7 ± 0.7	7.4 ± 5.3	51
m3-A	27	2.1 ± 0.6	8.4 ± 5.3	70
m3-B	102	4.5 ± 1.6	2.3 ± 2.6	27
m3-B(R)	39	1.4 ± 0.6	9.2 ± 6.7	25
m3ΔA	21	NR	NR	27
m3-C	26	6.5 ± 3.1	1.6 ± 1.8	155
m3ΔLM	126	2.7 ± 1.1	5.7 ± 4.0	39
m3ΔLV	60	4.6 ± 2.0	2.8 ± 3.1	98
m3ΔLK	59	6.4 ± 3.2	1.5 ± 1.7	61
m3ΔLM-C	25	7.8 ± 3.0	1.0 ± 0.6	50

Receptor expression levels are given as fmol NMS bound/100 μg protein. NR, no response to agonist.
[a]Number of oocytes from which recordings were made.

was expressed on the cell surface and competent to bind muscarinic ligands (Table I). Therefore, normal G protein signaling by the m3 mAChR requires a small number of basic residues which must reside ~9 amino acids away from the proposed cytoplasmic terminus of the fifth transmembrane domain; furthermore, within this charged sequence, a single arginine may satisfy the requirement (Figure 1).

In addition to the charged and polar sequences found near the amino-terminal region of the third cytoplasmic domain, all five mAChR subtypes, and most other G protein-coupled receptors, share a number of basic residues near the carboxy-terminal end of this domain (Peralta *et al.*, 1987; Seeman, 1992). Therefore, we examined whether this sequence also plays a significant role in G protein signaling by the m3 mAChR. Functional analysis of the substitution mutant termed m3-C, in which charged residues near the carboxy-terminus were converted to uncharged residues, revealed that these sequences are required for normal signaling by the m3 mAChR (Figure 2 and Table I). Carbachol-induced I-Cl responses in oocytes expressing m3-C receptors exhibited a long delay in their time to onset, as well as significant reductions in peak magnitudes. Ligand binding studies confirmed that the m3-C receptor was competent to bind agonist and expressed at levels comparable with the wild type m3 mAChR (Tables I and II). Therefore, substitution mutagenesis of the third cytoplasmic domain indicates that G protein signaling by the m3 mAChR requires a second cluster of charged residues adjacent to the sixth transmembrane segment.

Minimal m3 receptors define cytoplasmic residues as essential and sufficient for signal transduction

The previous mutagenesis experiments revealed the importance of a relatively small number of charged residues within the third cytoplasmic domain of the m3 mAChR. However, among members of the mAChR family, as well as several other G protein-coupled receptors for α-adrenergic, serotonergic and dopaminergic agonists, this domain is very large, ranging in length from ~70 to >200 residues (Seeman, 1992). Therefore, we wished to determine whether any other sequences within this domain are required for G protein activation by the m3 mAChR. To test this possibility, we constructed a series of 'minimal' receptors in which the 239 residue third cytoplasmic domain was reduced to as few as 15 amino acids (Figure 1). All four

Table II. Dissociation constants (K_D) for antagonist (NMS) and agonist (carbachol) binding to wild type and mutant m3 mAChRs expressed in transfected 293 cells

Receptor	Sites/cell ($\times 10^{-3}$)	K_D	
		NMS (pM)	Carbachol (μM)
m3	340 ± 20	220 ± 48	3 ± 1
m3-A	650 ± 10	190 ± 20	3 ± 1
m3-B	160 ± 6	120 ± 16	5 ± 2
m3-B(R)	145 ± 2	160 ± 9	50 ± 7
m3ΔA	710 ± 10	180 ± 17	3 ± 1
m3-C	2800 ± 60	310 ± 33	13 ± 4
m3ΔLM	170 ± 2	190 ± 12	16 ± 7
m3ΔLV	1770 ± 30	230 ± 21	5 ± 2
m3ΔLK	2010 ± 40	220 ± 19	6 ± 3
m3ΔLM-C	470 ± 4	190 ± 8	9 ± 4

m3-B(R) data were obtained by transient expression; all other assays were conducted with stably transfected cell lines.

minimal m3 receptors displayed ligand binding properties that were very similar to the wild type m3 mAChR; therefore, removal of the majority of the cytoplasmic domain did not appear to affect the normal processing and expression of the receptor (Table I).

We initially analyzed signal transduction by a minimal receptor, termed m3ΔLM, in which the first 12 residues of the cytolasmic domain are joined to the 10 residues adjacent to the sixth transmembrane segment. The m3ΔLM receptor contains all the charged sequences examined by substitution mutagenesis, as well as a short uncharged sequence proximal to the carboxy-terminus of this domain (Figure 1). Oocytes expressing the m3ΔLM receptor displayed large and rapid carbachol-induced I-Cl currents, very much like those generated by the wild type m3 receptor (Figure 2 and Table I). Elimination of three uncharged residues near the carboxy-terminal end of the third cytoplasmic domain within the m3ΔLV receptor resulted in carbachol-dependent I-Cl responses that were significantly slower in onset and smaller in amplitude compared with normal m3 mAChR-induced response (Figure 2 and Table I). However, removal of four additional amino acids, including the carboxy-terminal basic region shown to be important for signaling within the context of the full-length m3 mAChR, drastically reduced the ability of the m3ΔLK receptor to activate G protein signaling

(Figure 1). Agonist treatment of oocytes expressing the m3ΔLK receptor evoked extremely weak, irregular I-Cl responses which were substantially delayed in onset (Table I and Figure 2). Finally, to test the possibility that the weak m3ΔLK responses resulted from a general limitation in the size of the cytoplasmic domain (15 amino acids) rather than the absence of necessary activating sequences, we constructed the m3ΔLM-C receptor in which the cytoplasmic domain was restored to the size of the fully active m3ΔLM receptor. The m3ΔLM-C receptor contains a cytoplasmic domain of 22 residues in which the amino-terminal charged and polar sequences are joined to the same 10 uncharged residues found in the m3-C substitution mutant receptor (Figure 1). Despite the increase in the length of the third cytoplasmic domain, agonist treatments of oocytes expressing m3ΔLM-C evoked delayed and weak I-Cl responses similar to those generated by the m3ΔLK receptor (Figure 2 and Table I). Therefore, this analysis demonstrated that efficient activation of the G protein pathway leading to the stimulation of PLC-dependent responses minimally requires only a few conserved, charged residues found near the boundaries of the third cytoplasmic domain of the m3 mAChR.

Discussion

By measuring the ability of substitution and deletion mutant derivatives of the m3 mAChR to activate PLC-dependent responses in RNA-injected *Xenopus* oocytes, we found that a small number of charged residues at the boundaries of the third intracellular domain are required and sufficient for activation of G protein signaling by this receptor. Immunoblot analysis demonstrates that *Xenopus* oocytes express the PTX-insensitive G protein, termed $G_{\alpha q}$ or $G_{\alpha 11}$, which couples the mAChR to PLC stimulation in mammalian cells (M.Dell'Acqua and E.G.Peralta, unpublished observation; Gutowski *et al.*, 1991; Taylor *et al.*, 1991; Berstein *et al.*, 1992). Therefore, functional studies in *Xenopus* oocytes are predictive of the m3 receptor's properties in mammalian cells (Lechleiter *et al.*, 1990; Wess *et al.*, 1990).

Through the substitution of hydrophobic amino acids for conserved charged or polar residues within the third cytoplasmic domain of the m3 mAChR, we tested the involvement of these residues in G protein signaling without disrupting the secondary structure or relative spacing of other sequences within this domain of the receptor. We found that the charged and polar character of the $Y_{255}KETE_{259}$ sequence proximal to the fifth transmembrane segment was not important for normal signal transduction by the substitution mutant receptor m3-A. In contrast, substitution mutagenesis of the adjacent $K_{260}RTKE_{264}$ sequence demonstrated that this region is important for activation of signal transduction by the m3 mAChR. The m3-B receptor, containing hydrophobic residues at positions 260–264, only weakly activated PLC-dependent responses in oocytes. Notably, restoration of just the arginine at residue 261 was sufficient to allow wild type signaling by the m3-B(R) receptor. Interestingly, derivatives of the m3-B receptor containing either a lysine or glutamic acid at position 261 exhibited slightly stronger responses than the parental m3-B receptor, however, these responses were still weaker than those generated by the wild type m3 mAChR (unpublished observations). Thus, optimal activation of G protein signaling by the m3 mAChR specifically requires an arginine at position 261, although other basic or acidic residues may weakly substitute for this amino acid.

Substitution mutagenesis studies revealed that a second group of charged residues located near the sixth transmembrane segment are also required for normal G protein signaling by the m3 mAChR. This basic-rich sequence is highly conserved among a variety of G protein-coupled receptors and has been shown previously to effect receptor–G protein interactions. For example, single amino acid substitutions within the corresponding region of the α1-adrenergic receptor cause agonist-independent activation of G protein signaling (Cotecchia *et al.*, 1990; Kjelsberg *et al.*, 1992). In addition, others have found that a peptide composed of the basic-rich sequences found near the sixth transmembrane domain of the α2 adrenergic receptor block the ability of the receptor to interact with its cognate G proteins (Dalman and Neubig, 1991). Therefore, it is possible that this receptor region directly contacts G proteins or interacts with a distinct G protein activating sequence.

Previous efforts to define receptor regions involved in G protein activation have focused on the abilities of certain peptides to stimulate the GTPase activity of various G proteins. Since the stimulation of GTPase activity occurs optimally in the presence of phospholipids, these peptides are thought to mimic an activating region found in G protein-coupled receptors (Higashijima *et al.*, 1988). Examples of GTPase-stimulating peptides include mastoparan, as well as peptides corresponding to sequences within the third cytoplasmic domain of the α2-adrenergic receptor and m4 mAChR (Higashijima *et al.*, 1988; Okamoto and Nishimoto, 1992). Each of these peptides is 12–19 residues in length and contains a number of basic residues that may form the charged surface of an amphipathic α helix (Higashijima *et al.*, 1983). In addition, permutations of the mastoparan peptide and amino acid insertions within other G protein activating peptides demonstrate that the relative orientation of basic residues within the proposed helix is important for GTPase stimulation (Okamoto *et al.*, 1990; Oppi *et al.*, 1992). Our results demonstrate that the m3 mAChR is able to activate G protein signaling efficiently with a minimal third cytoplasmic domain consisting of a small number of basic amino acids reminiscent of the cationic residues found in GTPase activating peptides. Although our experiments do not reveal whether the charged sequences within the third cytoplasmic domain adopt an α helical conformation, analysis of the m3ΔA deletion mutant suggests that putative activating sequences must be correctly positioned to mediate G protein signaling. Interestingly, analysis of certain GTPase activating peptides indicates that the positioning of basic residues is important to the activation of G proteins, but that an α-helical conformation is not a requirement for this activity (Fujimoto *et al.*, 1991; Voss *et al.*, 1993).

We have found that efficient activation of G protein signaling by the m3 mAChR requires a surprisingly small number of charged residues within the large third cytoplasmic domain. In fact, as long as these charged sequences are maintained, a receptor containing only 22 amino acids within this domain (m3ΔLM) is nearly as active in signaling as the wild type m3 mAChR. It is somewhat puzzling that among the many G protein-coupled neurotransmitter and peptide hormone receptors that activate PLC, there is very little sequence similarity within the third

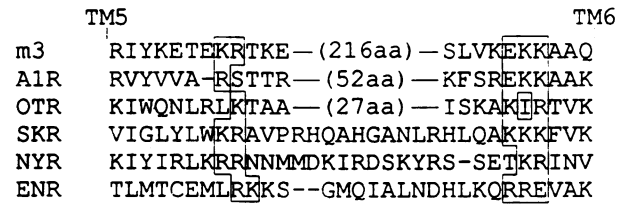

Fig. 3. Conservation of charged residues in the third cytoplasmic domain of G protein-coupled receptors that activate phospholipase C. Sequence alignment of the m3 muscarinic acetylcholine (m3), α1-adrenergic (A1R), oxytoxin (OTR), substance K (SKR), neuropeptide Y (NYR) and endothelin (ENR) receptors are adapted from Seeman (1992). Conservation of the charged residues shown to be important for G protein signalling by the m3 mAChR is indicated by boxes. Gaps in the sequence are indicated by dashes. The putative boundaries of the fifth (TM5) and sixth (TM6) transmembrane segments are indicated. The entire third cytoplasmic domain of the m3 muscarinic, α1-adrenergic and oxytoxin receptors is not shown; the number of additional amino acids (aa) contained in the domains of these receptors is indicated in parentheses.

cytoplasmic domain, moreover, the length of this domain varies drastically from ~30 to 240 amino acids. However, the important charged residues identified in our studies of the m3 mAChR are well-conserved in the corresponding regions of otherwise structurally disparate receptors (Figure 3). Thus, it is possible that these few charged residues play a similarly important role in G protein activation among widely divergent receptor families (Figure 3). Finally, it is interesting to note that although the majority of the third cytoplasmic domain is dispensable for activation of G protein signaling by the m3 mAChR, the entire domain is completely conserved across several species and may therefore serve a distinct receptor function. Our preliminary studies indicate that the wild type m3 and m3ΔLM receptors share similar cell surface distribution patterns when expressed in transfected NG108-15 neuroblastoma cells; both receptors are localized to cell bodies and neuronal processes (unpublished observation). Future studies will investigate whether the signaling-competent m3ΔLM receptor is altered in other properties such as agonist-induced desensitization.

Materials and methods

Oocyte electrophysiology

In vitro capped RNA was synthesized as previously described by Lechleiter *et al.* (1990) and Melton *et al.* (1984). Adult female *Xenopus laevis* were anesthetized in 3 g/l 3-aminobenzoic acid ethyl ester for 15 min and 1 or 2 ovarian lobes were removed, followed by immediate suturing of the incisions. Oocytes were defolliculated at room temperature with 5–10 mg/ml collagenase in Ca^{2+}-free medium (96 mM NaCl, 2 mM KCl, 5 mM HEPES and 1 mM $MgCl_2$ pH 7.5) for 1–2 h. Oocytes were stored at 19°C in HEPES-buffered 0.5 × L-15 medium pH 7.6 and injected with 50 nl RNA (~1 μg/μl) within 8 h after defolliculation (Lechleiter *et al.*, 1990). Before recording, injected oocytes were stored at 19°C in medium containing gentamicin at 50 μg/ml for 2–3 days with daily media changes.

A two-electrode voltage clamp (Oocyte clamp OC-725, Warner Instrument Corp., Hamden, CT) was used to measure agonist-induced currents from individual oocytes. Electrodes were pulled to resistances of 0.2–1 MΩ and filled with 3 M KCl. Recordings were made at room temperature from oocytes clamped at −70 mV in OR-2 solution (1.8 mM $CaCl_2$, 96 mM NaCl, 2 mM KCl, 5 mM HEPES and 1 mM $MgCl_2$ pH 7.4) using a saturating dose of carbachol (100 μM). Current records were stored and analyzed using PCLAMP version 5.6 (Axon Instruments, Foster City, CA). Carbachol did not evoke any detectable responses in water-injected control oocytes (data not shown). The m3ΔA receptor failed to respond to carbachol except in one oocyte pool in which mutant and wild type receptor responses were several times larger than those obtained in other experiments; the data obtained from this experiment were not included in this study. Within the same batch of oocytes, current responses of mutant receptors were always compared with the responses generated by the wild type m3 mAChR.

Cell culture and ligand binding

Human embryonic kidney 293 cells were transfected by the calcium phosphate method and stable clones were obtained as described by Lechleiter *et al.* (1990). Receptor numbers and antagonist K_D values were determined by Scatchard analysis with [^{3}H]*N*-methyl scopolamine (NMS) on intact cells as described by Peralta *et al.* (1987). Membranes from 293 cells were obtained as described by Dell'Acqua *et al.* (1993). 100–200 μg of membrane protein were incubated in buffer (20 mM Tris–Cl pH 7.4, 150 mM NaCl and 5 mM $MgCl_2$) with ligands for 1 h at room temperature in a 1 ml volume. In agonist displacement assays, the concentration of NMS used was 0.5 times the K_D of NMS as determined by Scatchard analysis. Non-specific binding was determined by the addition of excess atropine. Membrane binding assays were terminated by the addition of 2 ml ice cold PBS and immediate filtration through pre-wetted Whatman GF/F filters. Filters were then washed twice with 2 ml ice cold PBS, collected and counted. Carbachol displacement was best fitted by a one site model using the LIGAND program (Munson and Rodbard, 1980).

Oocytes were washed in a large volume of chilled HME (20 mM HEPES pH 8, 2 mM $MgCl_2$ and 1 mM EDTA) and resuspended in 1 ml HME supplemented with leupeptin and aprotinin at 10 μg/ml. Cells were lysed by 30 strokes of a Dounce homogenizer on ice followed by 15 s microcentrifugation at 10 000 r.p.m. The pellet was rehomogenized as above and the two supernatants were pooled. Membrane proteins in the supernatant were pelleted by centrifugation at 100 000 *g* (38 000 r.p.m., Beckman Ti70.1) for 1 h at 4°C. The supernatant was immediately removed and the membrane pellet was resuspended at a protein concentration of 5–15 mg/ml in HME. Saturating doses of NMS were used for receptor filter binding assays with oocyte plasma membrane proteins as described above.

Mutagenesis

Mutants of the m3 mAChR were constructed using synthetic oligonucleotides and single stranded plasmid templates as described by Kunkel *et al.* (1987). All mutations were confirmed by DNA sequencing (Tabor and Richardson, 1987).

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