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α-Helical distorting substitutions disrupt coupling between m3 muscarinic receptor and G proteins

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Acetylcholine stimulation of the m3 or m2 muscarinic receptor expressed in *Xenopus laevis* oocytes induces either a fast transient or slowly oscillating calcium-sensitive chloride current. The speed of these currents reflects the efficiency of receptor coupling to guanine nucleotide-binding proteins and phosphatidylinositol (PI) turnover. Point mutations of the m3 receptor were made in a region of the third cytoplasmic loop to test whether receptor function relied on an α-helical structure of the G protein-coupling domain. Proline substitution for glutamate at position 257 disrupted the m3 response. Also, single alanine insertions between residues 259 and 260 disrupted the m3 receptor-stimulated response while double alanine insertions at this site had no effect. Based on these results, we suggest that a region of the third cytoplasmic loop of the m3 receptor possesses an amphipathic α-helical conformation.

Xenopus oocyte; G protein-coupling, Amphipathic α-helix; Seven transmembrane-spanning receptors, Mastoparan

1. INTRODUCTION

mAChRs are central to the regulation of many cellular responses including smooth muscle tone, heart rate, secretion, synaptic transmission, neuronal excitability, cognition and memory [1]. Genomic cloning revealed there are at least five distinct human gene sequences for the mAChR designated as subtypes m1–m5* [2–5]. Biochemically, the mAChR subtypes belong to one of two classes. Subtypes m1, m3 and m5 stimulate turnover of PIP_2, liberating the intracellular second messengers $Ins(1,4,5)P_3$ and diacylglycerol [6]. $Ins(1,4,5)P_3$ triggers the release of Ca^{2+} from intracellular stores while diacylglycerol stimulates protein kinase C activity [7]. Receptor subtypes m2 and m4 inhibit adenylyl cyclase and weakly stimulate PIP_2 turnover [8–10]. These biochemical systems are a feature shared by many neurohormonal cell membrane receptors which effect signal transduction by stimulating, in a very specific fashion, a family of heterotrimeric G proteins [11]. A central question of signal transduction for neurohormonal agents is the molecular mechanisms regulating the receptor's specificity for these different G proteins. Structural analysis of the mAChRs indicate that they have a hydropathic profile similar to that of other G protein-linked receptors, identifying them as a member of this superfamily [3]. Inference from the crystal structure of bacteriorhodopsin, which possesses a hydropathic profile similar to this receptor superfamily, predicts that the mAChRs have seven transmembrane-spanning regions connected by three extracellular and three cytoplasmic loops. Previous studies have implicated numerous cytoplasmic regions as contributing to G protein coupling for different G protein-linked receptors [12]. However, the precise molecular details of the interaction between these receptors and their G proteins is not clear, preventing a complete understanding of the mechanisms regulating signal transduction for hormones and neurotransmitters.

In order to study cell membrane receptors regulating $Ins(1,4,5)P_3$ production and intracellular Ca^{2+} release on a fast time scale, Lechleiter et al. [9] employed transient expression of exogenous membrane proteins in *Xenopus* oocytes. The endogenous $I_{Ca\text{-}Cl}$ in these oocytes is easily measured and provides a sensitive indicator of $Ins(1,4,5)P_3$ production. For example, following stimulation of *Xenopus* oocytes expressing the m3 AChR, a fast transient $I_{Ca\text{-}Cl}$ was observed [9]. 96% of these desensitizing (D1 type; [9,13]) currents peaked within the first 20 s of receptor stimulation ($n = 454$). Muscarinic receptor subtype 2 (m2) evoked currents which displayed

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Abbreviations. $I_{Ca\text{-}Cl}$, calcium-sensitive chloride current, D1 type, fast desensitizing current; f type, slowly developing oscillatory current; G proteins, guanine nucleotide-binding proteins; mAChR, muscarinic acetylcholine receptor; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; $Ins(1,4,5)P_3$, D-myo-inositol 1,4,5-trisphosphate; S.E.M., standard error of the mean; PKC, protein kinase C.

*Subtypes of the mAChR are designated as m1, m2, m3, m4 and m5 as defined by the Fourth International Symposium on Subtypes of Muscarinic Receptors.

a clearly different profile. Oocytes expressing the m2 AChR generally exhibited a slowly developing and fluctuating (f type; [13]) current, 72% of which peaked between 20 and 120 s ($n = 451$; [9]). The distinct kinetics of the D1 and the f type currents are thought to represent separate transduction pathways for Ins(1,4,5)P_3 release. Dual pathways are supported by several lines of evidence. For example, comparable levels of expression and agonist binding behavior were observed for these mAChRs in eukaryotic cells suggesting that the different kinetic profiles for the currents were not due to differences in receptor expression. The independence of the latency-to-peak current as a function of $I_{Ca\text{-}Cl}$ amplitude and the differential sensitivity of the D1 and f currents in oocytes to inhibition by pertussis toxin (PTX) further support dual signalling pathways. To address the question of which cytoplasmic domains of the receptor specify the type of G protein activated, Lechleiter et al. [9] assayed several m2/m3 chimeric receptors and characterized their responses according to the currents that they evoked. Their results indicated that a major determinant of receptor-G protein coupling was localized to the amino terminus of the third cytoplasmic loop [9]. Contrary to what has been observed for several G protein-linked receptors, other regions, such as the carboxy terminus of the third cytoplasmic loop or the carboxy tail of the mAChR, to date, have not been implicated in the initial response ([9] and unpublished results). The structural requirements relating to receptor function, however, are unknown.

Recently, it was reported that several amphipathic α-helical peptides could stimulate G proteins [14]. Most notably, the tetradecapeptide wasp venom, mastoparan, stimulated in vitro GTP binding and hydrolysis with a variety of purified G proteins. It is well established that amphipathic α-helical motifs frequently regulate protein-protein interactions [15]. Comparison of the G protein activating potential of mastoparans [14] to membrane-associated cell signalling suggest that cytoplasmic regions of the Ins(1,4,5)P_3-linked receptors regulating G protein activation might possess α-helical properties as predicted from various protein secondary structure algorithms [9,16,17]. The following report examines this hypothesis by placing point mutations in key sites of the G protein interactive domain of the m3 receptor and assaying for production of either the D1 or f type currents.

2. MATERIALS AND METHODS

Plasmids containing the cloned human m3 and m2 AChR [3], were used for production of mRNA using a standard SP6 driven in vitro transcription protocol [18]. Point mutants of the human m3 receptor were made using oligonucleotide-directed mutagenesis techniques [19]. Mutated phagemid constructs were verified by DNA chain termination sequencing [20].

Techniques for injecting and recording from oocytes were reported previously [9] Briefly, oocytes were defolliculated to reduce endogenous ACh responses and subsequently injected with approximately 50 ng of mRNA. m2, m3 AChR mRNA- or deionized H_2O-injected oocytes were examined in parallel with mutant receptor mRNA to control for oocyte variability in current responses. Injected oocytes were stored overnight in L-15 supplemented medium, changed daily. Current measurements were performed 2 days post-injection. Currents were evoked following addition of 50 μM ACh to the media (Barth's) bathing oocytes and voltage-clamped at a holding potential of −70 mV.

Infrequently, ACh stimulation of mRNA-injected oocytes evoked either small or no $I_{Ca\text{-}Cl}$. Because of H_2O-injected oocytes often times would evoke small amplitude currents it was difficult to establish whether currents below 100 nA were due to the expression of injected mRNA or due to endogenous mechanisms for evoking $I_{Ca\text{-}Cl}$. Therefore, only those mRNA-injected oocytes evoking peak currents greater than 100 nA were retained for comparison. This resulted in the exclusion of a small number of measurements (excluded/total; m3: 2/39; m2: 4/32; E257P: 7/37; T258P: 0/45, E257A 0/18; EK259EAK 1/18; and EK259EAAK: 0/19).

3. RESULTS

Stimulation of m3 receptors expressed in *Xenopus* oocytes with a saturating concentration of acetylcholine (ACh, 50 μM) uniformly produced a large desensitizing (D1 type) current (4,700 ± 600 nA, S.E.M.; Fig. 2). These oocytes attained a peak current rapidly (2 ± 1 s, S.E.M.) with a near complete return to baseline within 20 s in 32 of 37 oocytes. Stimulation of m2-expressing oocytes, however, evoked slowly developing fluctuating (f type) currents peaking between 20 and 120 s in 22 of 28 oocytes. The average latency to peak current in these oocytes was significantly longer than observed with stimulation of m3 AChRs (56 ± 12 s, S.E.M.; Fig. 2). All mRNA-injected oocytes responses were compared to H_2O-injected oocytes to control for endogenous ACh responses (see section 2). The H_2O-injected oocytes responded poorly to ACh, generally evoking either a small or no f type current (average peak $I_{Ca\text{-}Cl} < 100$ nA, $n = 28$). Since $I_{Ca\text{-}Cl}$ recordings provided a high time resolution (ms) measure of changes in intracellular calcium concentrations [10], which correlated well with Ins(1,4,5)P_3 production [8,21], the D1 and f type currents were used to assay for G protein coupling-ability of mutant m3 receptors.

Variants of the m3 AChR, containing mutations in a region of the receptor known to regulate coupling to G proteins, were assayed for their ability to preserve or alter the normal signal transduction pathways. These mutants involved either substitution of residues with proline, in order to interrupt putative α-helical structure, or insertions of alanine, designed to disrupt the spatial distribution of charges around a helix. ACh stimulation of a proline-substituted mutant m3 AChR, T258P (Fig. 1), evoked a fast transient current resembling the D1 type current (Fig. 2). In spite of the generally lower current amplitude for mutant T258P, perhaps due to decreased receptor expression or less effective PI coupling, 38 of 45 oocytes exhibited current peaks during the initial 20 s (17 ± 4 s). Proline did not alter the

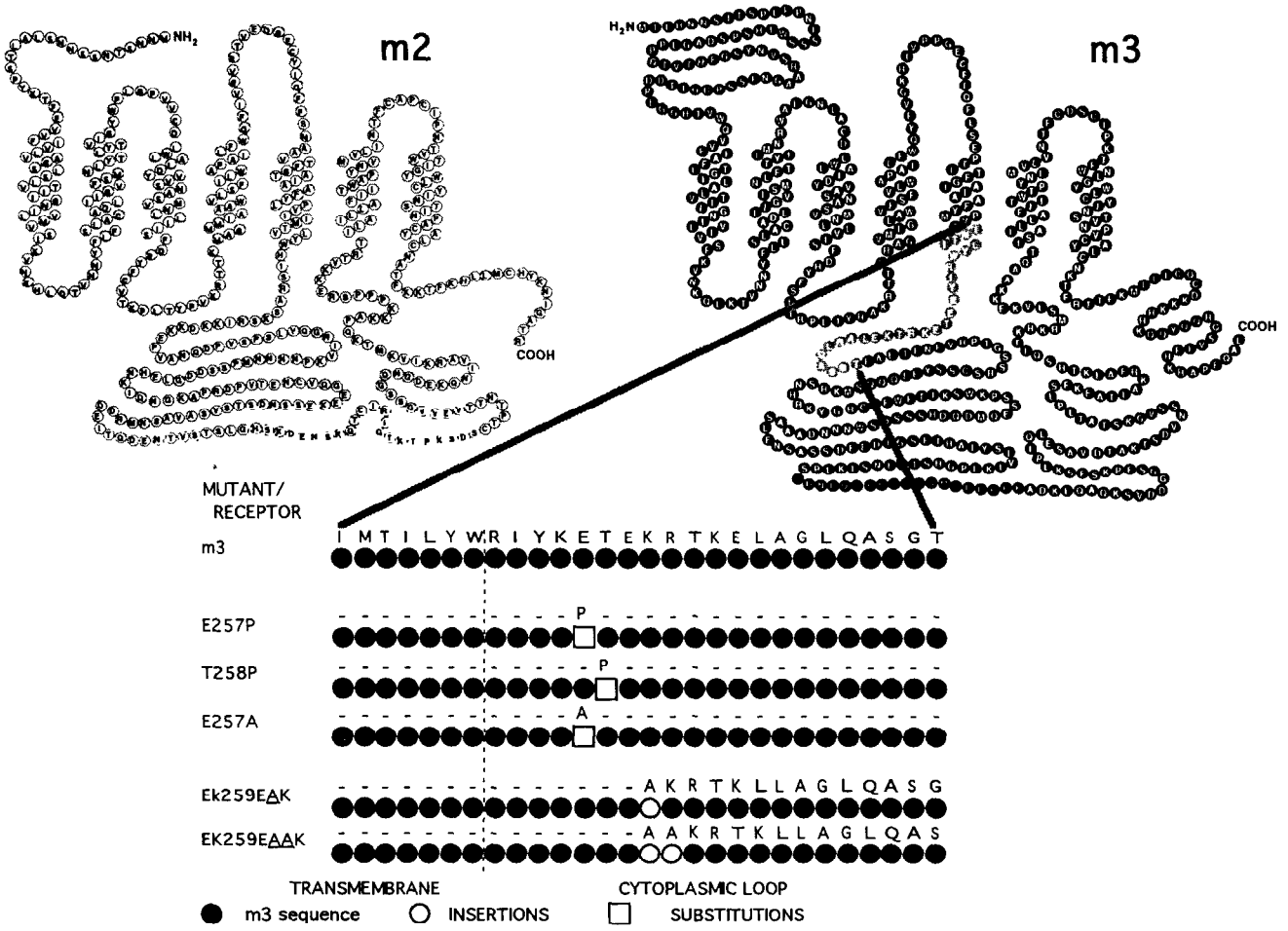

Fig. 1. Structural models of parental and mutated mAChRs. Putative transmembrane models for the m2 and m3 muscarinic receptor subtypes are illustrated (top). The greatest heterogeneity across the mAChR family is found at the amino terminus, the large third cytoplasmic loop and the carboxy terminus [3]. A region of the m3 receptor known to mediate receptor–G protein coupling, as well as the position and corrected amino acid sequence of several point mutants, is shown on an expanded scale. Individual residues are represented by the one letter amino acid code Dashes indicate amino acids identical to the corresponding position of the m3 receptor. Vertical dashed line represents the transition from the fifth transmembrane-spanning segment to the third cytoplasmic loop.

kinetic profile of the m3 AChR-mediated response when substituted at this position. Substitution of proline for glutamic acid at the adjacent residue, E257P (Fig. 1), however, resulted in agonist-evoked f type currents (Fig. 2). Of 30 oocytes expressing mutant E257P, 25 peaked later than 20 s (73 ± 7 s, S.E.M.). An alanine-substituted mutant, E257A (Fig. 1), was assayed to test whether the presence of a negatively charged amino acid at this glutamic acid residue was critical to development of the D1 type current. Receptor activation of E257A consistently evoked the D1 type response in 18 oocytes (2 ± 1 s, S.E.M.; Fig. 2). Thus, there is a marked contrast in the response between substitution of an alanine and a proline at residue 257. The D1 type response elicited by stimulation of E257A indicates that the loss of the negatively charged glutamate along cannot account for the disruption of the D1 type current exhibited by E257P.

We designed and assayed several insertional mutants which were predicted to distort any amphipathic α-helical character for the amino terminal region of the third cytoplasmic loop (Fig. 1). Of the naturally occurring amino acids, alanine insertions are energetically the most compatible to α-helical formation [22,23]. Therefore, insertion of an alanine would be expected to influence the hydrophobic/amphipathic nature for this region of the receptor while preserving its α-helical structure. Twelve of 17 oocytes expressing the alanine insertional mutant EK259EAK (Fig. 1) exhibited f type currents (57 ± 10 s, S.E.M.; Fig. 2B). It is unlikely that EK259EAK failed to evoke the D1 type current due simply to an extended primary structure for this region of the receptor since a double alanine insertional mutant, EK259EAAK (Fig. 1), resulted in the D1 type current. Sixteen of 19 EK259EAAK expressing oocytes responding within the first 20 s of ACh exposure (15 ± 5 s,

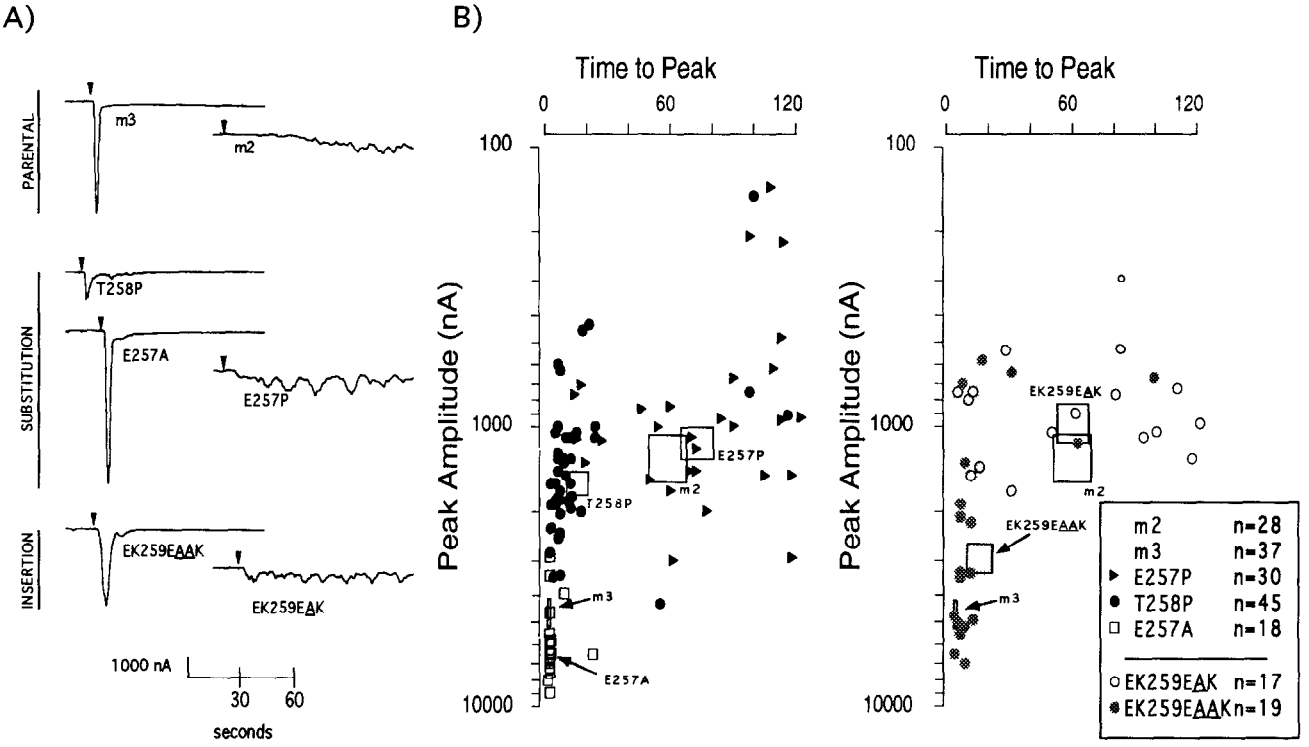

Fig. 2. Parental and mutant mAChR demonstrate distinct Ca^{2+}-activated chloride currents ($I_{Ca\text{-}Cl}$) in *Xenopus* oocytes (A) Representative current waveforms are shown for the m3, m2 and mutated m3 receptors Single amino acid substitutions were made at either glutamate (E) 257 or at threonine (T) 258. An additional pair of mutants containing single or double alanine (A) insertions following glutamate (E) 259 were also examined (see Fig. 1). 50 μM ACh was added at the arrow. The fast transient (D1) current following stimulation of the m3 receptor is differentially sensitive to mutations of the m3 receptor at the indicated residues (see also text). (B) The latency to peak and maximum amplitudes are displayed for the currents from individual receptor-expressing oocytes (see inset at right). The shaded region between 0 and 20 s represents the period containing 96% of the peak amplitudes for the m3-stimulated D1 currents [9]. The average ± S.E.M for both measurements is indicated by a box

S.E.M.; Fig. 2B). These results are evaluated below in the context of the spatial positioning of amino acids in an α-helical model.

4. DISCUSSION

The amino terminus of the third cytoplasmic loop of the mAChR is recognized as the major determinant specifying coupling between these receptors and G proteins [9,24]. When expressed in *Xenopus* oocytes, the m3 and m3 AChR elicit a D1 or f current, respectively. Similar characteristic kinetic profiles were also observed for several mAChR variants containing mutations of the third cytoplasmic loop [9]. The ability to elicit a unique pattern of $I_{Ca\text{-}Cl}$ in oocytes for these mAChRs, which exhibit comparable receptor expression and agonist binding behavior in eukaryotic cells, supports a role for dual signalling pathways in evoking either the D1 or f currents [9]. We have used this system, therefore, to test whether the efficacy of G protein-coupling to m2 receptors requires an amphipathic α-helix in this region of the receptor.

A marked difference was observed between proline substitution at adjacent amino acid residues (E257P vs T258P; Fig. 1). Although agonist stimulation of the distally located substitution mutant, T258P, resulted in D1 type current, a more membrane proximally located mutation, E257P, produced a nondesensitizing f type current. The inability of the latter to signal to the D1 pathway cannot result from changes in the receptor's electrostatic properties since alanine substitution at the same position (E257A) also elicited D1 type currents. Proline is energetically incompatible with α-helices [22,23,25,26] and we interpret these results as evidence of the conformationally induced inability of E257P to interact with the G protein normally associated with the m3 AChR. However, this interpretation is probably a simplification given the ability of T258P to elicit the D1 type currents. Examination of m2/m3 AChR chimeras suggested that amino acids proximal to the fifth transmembrane segment (Arg-Iso-Tyr-Lys; RIYK) partly specify the coupling of m3 receptors to G proteins (compare HY12 and 22, [9]) but the exact residue(s) defining this G protein-coupling region are uncertain. Our results indicate that proline substitution immediately adjacent to amino acids RIYK has a greater disruptive influence on the m3 receptor-G protein signalling pathway than at the more distally located threonine.

Alanine insertional mutants were assayed to test whether the amphipathic nature of the α-helix influ-

enced receptor-G protein coupling. Helical wheel models for m3 and the insertional mutant receptors demonstrated varying degrees of charge separation (Fig. 3). Mutant EK259EAK exhibits no distinct amphipathic character and we believe that this property is reflected in the f type currents elicited by this mutant. Modeling of mutant EK259EAAK, which elicited the D1 currents representative of a normal signalling pathway, shows a separation of charged surfaces intermediate between that of EK259EAK and the m3 AChR. A correlation between the amphipathic character for this region of the m3 receptor variants and their ability to mimic the normal receptor-G protein signalling is further strengthened by two points. First, the terminal amino charge of lysine (K) is separated from the peptide backbone by a 4 hydrophobic methylene groups. These flexible linkers may permit a partial dispersion of the terminal amino charge for those lysines bordering the hydrophobic surface in our model [22]. This property results in a less dramatic intrusion of those charges defining the boundary between the charged and uncharged surfaces and, therefore, a more definite amphipathic characterization. Second, the region containing these charges is several turns of the α-helix distal to RIYK, which is reported to participate in G protein coupling (see above). Regions located distal to this site, therefore, may minimally influence protein–protein interactions occurring at these residues simply by nature of their displacement along the axis.

Attempts to explain the results of the insertional mutants based on a model of linear displacement of the primary structure simply would not be consistent with the observed recordings. Although such a model might be reflected in the disruption of the receptor–G protein interaction generating the D1 currents, as exhibited by mutant EK259EAK, it is clearly inappropriate to explain the unaltered current kinetics elicited by mutant EK259EAAK (Fig. 2).

Although it is clear that only structural data, such as X-ray diffraction of the receptor-G protein complex, can determine which regions are directly involved in protein–protein interactions, site-directed mutagenesis

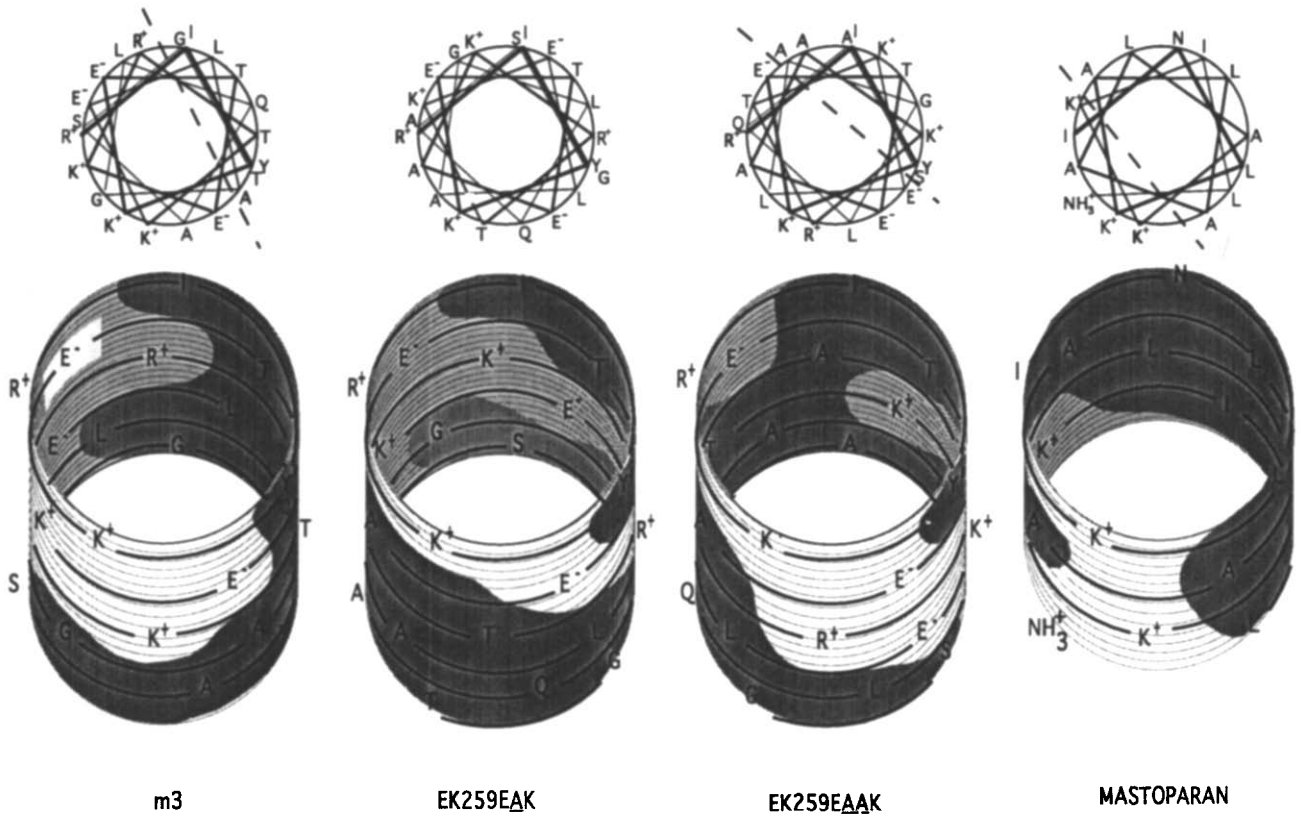

Fig. 3. Helical wheel models representing the amino terminus of the third cytoplasmic loop for the m3 and insertional mutant receptors. Individual residues are represented by the one letter amino acid code. The positively charged arginine, seen at the top of the helix in the three dimensional model, is the putative first amino acid following the fifth transmembrane-spanning segment. The darkly shaded regions (second row) indicate surfaces composed of uncharged amino acid residues. A clear charge separation is exhibited in the model for the m3 receptor. Modeling for the double alanine insertional mutant EK259EAAK indicates that it is intermediately amphipathic (see text for details). Mutant EK259EAAK contains a glutamate (E) and an arginine (R) imposed on the uncharged surface of the wild type receptor resulting in an even less well defined charge separation. The G_α-stimulating amphipathic peptide mastoparan is shown on the right for comparison. The separation of charged and uncharged surfaces is also indicated by a dashed line in the 2 dimensional helical wheel models in the first row.

provides important information on the functional consequences of receptor alterations. Our results presented here support the simple hypothesis that the amphipathic α-helical structure of the amino terminal region of the third cytoplasmic loop is a critical determinant for G protein coupling by the m3 AChR.

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