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Alanine Scanning Mutagenesis of Conserved Arginine/Lysine-Arginine/Lysine-X-X-Arginine/Lysine G Protein-Activating Motifs on m1 Muscarinic Acetylcholine Receptors

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SUMMARY

Alanine scanning mutagenesis of B-B-X-X-B motifs (where B is a basic residue and X is any nonbasic residue) in m1 muscarinic acetylcholine receptors was performed to determine the relative roles of basic amino acids in receptor coupling. This conserved motif is found in many G protein-coupled receptors and has been implicated in G protein activation. The KKAAR365 motif, located at the carboxyl-terminal third intracellular loop of m1 receptors, was mutated to AAAAA365, thereby generating a triple-substitution mutant devoid of ability to stimulate either phosphoinositide (PI) hydrolysis or cAMP accumulation. In contrast, a triple-alanine substitution of the KRTPR140 motif in the carboxyl-terminal second intracellular loop, yielding mutant AATPA140, had no effect on receptor coupling to the two independent second messenger pathways. Analysis of a series of single- and double-substitution mutants demonstrate that all three basic residues of the KKAAR365 motif participate in efficient m1 receptor coupling. The presence of second and third basic residues in this motif was absolutely critical for full agonist recognition of a high and low affinity state of the receptor. Mutation of either Lys362 or Lys365, but not Lys361, abolished guanine nucleotide-dependent conversion of agonist affinity states and correlated with an inability of full agonists to fully activate PI hydrolysis. The different combinatorial double-substitution mutants also revealed that Lys365 was necessary but not sufficient, in the context of the KKAAR365 motif, for efficient receptor coupling. This residue cannot facilitate full agonist-stimulated PI hydrolysis in the absence of both Lys361 and Lys362. In comparison, the critical residue Lys362 was both necessary and sufficient. Substitution of nearby basic residues Lys361 and Lys365 with alanine yielded mutant AKAAA365, which exhibited partial ability to couple PI hydrolysis after full agonist stimulation. Therefore, Lys365 seems to function in a hierarchal (interdependent) manner with nearby basic residues, whereas Lys361 and Lys362 can act independent of surrounding basic residues to facilitate partial m1 receptor coupling after full agonist stimulation. In contrast, all three residues must be present for stimulation of PI hydrolysis by a partial agonist.

A diverse group of neurotransmitters, peptide hormones, and regulatory factors are known to control cellular physiology through their interaction with integral membrane receptors coupled to heterotrimeric G proteins. The molecular mechanism by which receptor proteins regulate second messenger pathways via G proteins has been under intensive investigation (1). The vast majority of G protein-coupled receptors belong to a gene superfamily with each member sharing a common putative configuration of 7TMD (for reviews, see Refs. 2 and 3). Prototypical examples include the mAChR and β-adrenergic receptors. Examples also exist of receptors that are structurally unrelated to 7TMD receptors yet functionally interact with G proteins. Recently, the insulin-like growth factor II/mannose 6-phosphate receptor and the β-amyloid precursor protein, both containing a single transmembrane domain, were shown to directly activate the G proteins G_i and G_o, respectively (4–6).

Insights pertaining to the linkage mechanism between receptors and G proteins have been forthcoming. Based on deletion mutation and chimeric receptor analysis, it seems that multiple intracellular domains found on 7TMD receptors determine the specificity of receptor interaction and activation of G proteins (3, 7–10). These domains include the i-2 loop, the amino- and carboxyl-terminal domains of the i-3 loop, and the membrane proximal portion of the carboxyl tail. Synthetic peptides of these receptor domains have also been shown to mimic receptor/G protein interactions by stimulating GTP binding (11) or inhibiting receptor coupling to G proteins (12, 13). The sites of G protein interactions with their cognate receptor have also been delineated. Several lines of evidence suggest that the carboxyl terminus of the G_α subunit of G proteins is an important site of interaction with 7TMD receptors (14).

Site-directed mutagenesis has served as a useful model to

ABBREVIATIONS: 7TMD, seven transmembrane domain; CHO, Chinese hamster ovary; i-2, second intracellular; i-3, third intracellular; mAChR, muscarinic acetylcholine receptor; PI, phosphoinositide; QNB, quinuclidinyl benzilate; NMS, *N*-methylscopolamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

further delineate and identify some of the amino acids involved in the process of 7TMD receptor coupling to G proteins (for reviews, see Refs. 1, 3, and 15). The amino acids aspartate and arginine of the conserved aspartate-arginine-tyrosine (DRY) motif located at the amino-terminal i-2 loop of many 7TMD receptors are essential for efficient receptor coupling to diverse second messenger pathways (16–19). Also found in the i-2 loop (middle portion) is a solitary, highly conserved, bulky, hydrophobic residue (e.g., leucine, isoleucine, phenylalanine) that seems to be necessary for efficient G protein coupling (20). Other important amino acids include a tyrosine and an alanine residue located at the amino- and carboxyl-terminal i-3 loop, respectively, of m1, m3, and m5 mAChRs (21, 22). These residues apparently are critical for activation of the $G_{q/11}$ class of G proteins, which stimulate PI hydrolysis.

Less well defined are the amino acids on single transmembrane domain receptors that are involved in G protein activation. The G protein activation domain on the insulin-like growth factor II/mannose 6-phosphate receptor and β-amyloid precursor protein has been localized to a small 14–20-amino acid stretch of the cytoplasmic tail. Synthetic peptides corresponding to this region are able to stimulate GTP binding to various G proteins (4, 5). These peptides all have in common a B-B-X-X-B or B-B-X-B motif, where B is the basic amino acid arginine, lysine, or histidine, and X is any nonbasic amino acid. Of interest is the finding that the G protein-activating peptides derived from 7TMD receptors also contain this B-B-X-X-B motif. For example, synthetic peptides of 14–19 amino acids corresponding to the terminal i-3 loop segments of the β_2-adrenergic receptor and m4 mAChR stimulate G_s and G_i, respectively (11, 23, 24). Therefore, the B-B-X-X-B motif has been postulated to act as a "universal" G protein activation domain (24). Recent mutagenesis studies in the terminal i-3 loop portion of mAChRs provide indirect evidence supporting this hypothesis (22, 25–28). However, these studies describe deletion or multisubstitution mutations that partially encompass and span beyond the B-B-X-X-B motif, thereby making it difficult to address directly the nature of this motif in receptor coupling. Also, it is not known whether the individual basic amino acids in this motif contribute to receptor coupling in an additive, mutually exclusive, or hierarchal manner. To comprehensively examine the contribution of the B-B-X-X-B motif in receptor/G protein coupling, we performed alanine scanning mutagenesis of two motifs found on the carboxyl-terminal i-2 and i-3 loops of the m1 mAChR subtype. Single, double, and triple basic amino acid substitutions were obtained, and the relative role of each residue in the coupling of m1 mAChRs to two independent signaling pathways, PI hydrolysis and cAMP accumulation, are described.

Experimental Procedures

Materials. CHO-K1 cells were obtained from American Type Culture Collection (Rockville, MD). [^{3}H]QNB (36.4 Ci/mmol), [^{3}H]NMS (79.5 Ci/mmol), and *myo*-[^{3}H]inositol (20.5 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA). Carbachol, acetylcholine, oxotremorine, atropine, NaF, and GTP were obtained from Sigma (St. Louis, MO). The vector pGEM11 and *Escherichia coli* BMH 71–18 cells were purchased from Promega (Madison, WI), and the mammalian expression vector pcDNA3 was purchased from InVitrogen Corp. (San Diego, CA). Restriction endonucleases, Dulbecco's modified Eagle's medium, fetal calf serum, G-418, and *E. coli* DH5α cells were obtained from Life Technologies (Grand Island, NY).

Alanine scanning mutagenesis and expression of receptors in CHO cells. To study the functional role of the basic amino acids arginine and lysine in the G protein-activating motif B-B-X-X-B of m1 mAChRs, site-directed mutagenesis was carried out according to the method of Deng and Nickoloff (29) with slight modifications (30). Briefly, a 1.6-kb *XhoI/Bam*HI fragment of the m1 mAChR gene (16) containing the entire coding region was subcloned into the polylinker region of pGEM11 to yield Rm1pGEM11. Double-strand wild-type Rm1pGEM11 was denatured through heating and annealed to two synthetic oligonucleotides of 23 and 30 bases. The 23-mer is a selection primer (5′-GAGGTCGAC GATATCGAG CTCGG-3′; underlined bases are *Eco*RV site) that converts the restriction endonuclease site *Eco*RI to *Eco*RV in the polylinker region. A series of 30-mers were used as mutagenic primers in which a single arginine (CGC, CGA, or CGG) or lysine (AAG) codon in the m1 gene was converted through a double-base change to an alanine codon (GCC, GCA, or GCG). The mutant second strand of DNA incorporating both primers was synthesized with the use of T4 DNA polymerase and dNTPs. The resulting Rm1pGEM11 heteroduplex was transformed into the bacterial strain BMH 71–18 *mut S*, and the DNA from the transformants was pooled and digested with the restriction endonuclease *Eco*RI. Rm1pGEM11 mutants incorporating the 23-mer are not linearized with *Eco*RI treatment and thus can be amplified in DH5α bacterial cells. Colonies from these transformants were picked, and Rm1pGEM11 mutants were isolated. The authenticity of all mutations was confirmed through dideoxy sequencing before insertion of the mutant receptor genes into the expression vector pcDNA3. Mutant receptor gene constructs in pcDNA3 were individually transfected into CHO-K1 cells as described previously (31, 32).

To generate combinatorial arginine/lysine to alanine mutant m1 mAChR genes for the purpose of alanine scanning mutagenesis, mutant Rm1pGEM11 constructs containing the newly acquired *Eco*RV site in the polylinker region and single codon mutations were denatured and annealed to another set of primers. A 23-mer selection primer was used to convert the *Eco*RV site to *Eco*RI (5′-GAGGTCGACGAATTCGAGCTCGG-3′; underlined bases are *Eco*RI site), and a series of 30-mer mutagenic primers were used for incorporating multiple codon mutations in the m1 gene (Fig. 1). Rm1pGEM11 recombinants containing multiple codon mutations in the B-B-X-X-B motif were selected by treating pooled DNA isolated from BMH 71–18 *mut S* transformants with the endonuclease *Eco*RV and amplifying in DH5α cells.

Receptor binding and second messenger assays. Saturation binding assays with [^{3}H]QNB in plasma membrane homogenates and Scatchard analysis of binding data were performed as described previously (31, 32). Membrane homogenates (15–20 μg) are incubated with increasing concentrations of [^{3}H]QNB in HEPES buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$) for 60 min at 37°. Reactions, performed in duplicate, were terminated through rapid filtration through Whatman GF/C glass filters with a Brandel cell harvester. Specific binding is defined as the difference in [^{3}H]QNB binding in the absence and presence of 10 μM atropine. Agonist/[^{3}H]QNB or agonist/[^{3}H]NMS competition displacement curves in the absence and presence of 100 μM GTP were performed in plasma homogenates as described previously (33). Briefly, membrane homogenates (15–20 μg) are incubated with displacer, labeled antagonist (concentration equal to $2 \times K_d$ for each receptor) and with or without GTP in 20 mM HEPES buffer, pH 7.4. Reactions, performed in duplicate or triplicate, were carried out for 60 min at 37° and terminated through rapid filtration. Data from displacement curves were analyzed for goodness of fit to a one- versus a two-site model by using a computerized iterative nonlinear least-squares curve-fitting program (Inplot, GraphPAD, San Diego, CA) as described previously (31, 33).

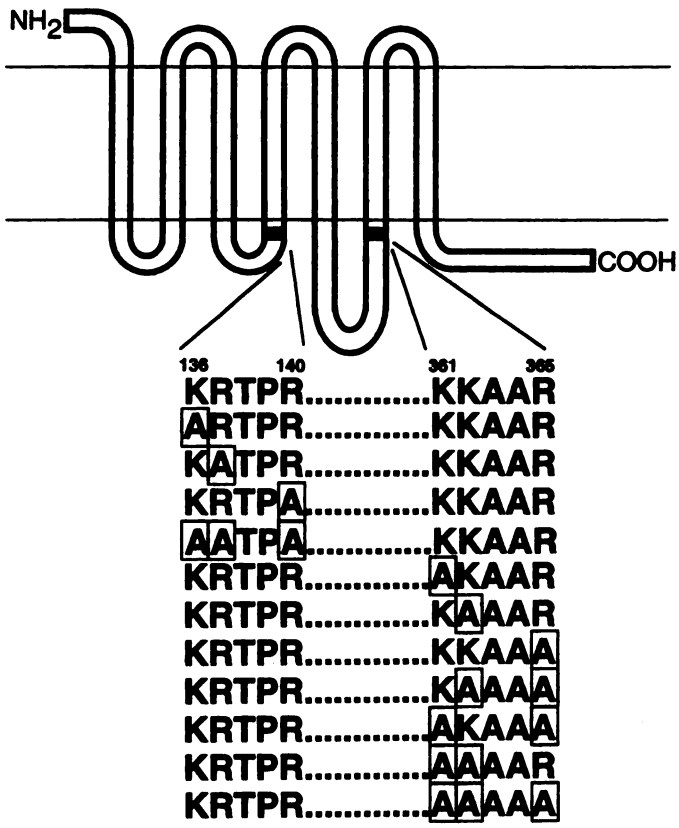

Fig. 1. Structure of the wild-type and single, double, and triple B-B-X-X-B substitution mutant m1 mAChRs. Two B-B-X-X-B motifs (B is any basic amino acid residue and X is any nonbasic residue) in the carboxyl-terminal i-2 and i-3 loops were subjected to alanine scanning mutagenesis. *Boxes*, the basic amino acid residues lysine (K) and arginine (R) in the wild-type receptor were substituted with alanine (A). A total of four i-2 loop and seven i-3 loop mutants were constructed and stably transfected individually into CHO cells.

Transfected CHO cells expressing wild-type and mutant m1 mAChRs were assayed for carbachol-stimulated PI hydrolysis and cAMP accumulation as described previously (17, 31). Dose-response curves were analyzed with the use of computerized iterative nonlinear least-squares regression (Inplot).

Results and Discussion

The B-B-X-X-B motifs in the carboxyl-terminal i-2 (KRTPR140; number indicates position of last amino acid) and i-3 loops (KKAAR365) of the m1 mAChR were subjected to alanine scanning mutagenesis, thereby generating a total of 11 mutant receptors (Fig. 1). Although two other B-B-X-X-B motifs exist on the internal portion of the i-3 loop, these sequences were not mutated in the current study inasmuch as i-3 loop deletions encompassing these motifs have been shown previously not to affect PI turnover (31, 34, 35). Because maximal agonist stimulation of PI hydrolysis is dependent on m1 mAChR expression levels in CHO cells (36), we picked stably transfected cell lines expressing levels of wild-type and mutant mAChRs of 0.5–2 pmol/mg membrane protein (Table 1). These expression levels for the wild-type receptor are known to exhibit equivalent PI hydrolysis responses to agonists (36). We used stably transfected clones over transient transfection protocols because consistent detection of differences in receptor coupling efficiency is best achieved by having a constant, well-defined number of expressed receptors.

Mutant m1 mAChRs displayed high affinity, saturable binding of the antagonist [^{3}H]QNB, with K_d values of 35–103 pM. These values are comparable to that obtained for the wild-type receptor (K_d of [^{3}H]QNB binding to wild-type receptor = 71 pM; see Table 1). Although antagonist binding parameters of mutant receptors were equivalent to those of wild-type, distinct differences in agonist binding profiles were observed (Fig. 2 and Table 1). Wild-type m1 mAChRs stably transfected into CHO cells are known to exhibit two affinity states for the full agonist carbachol in the absence of guanine nucleotides (31, 33). In the absence of GTP, only the AATPA140, AKAAR365, and KKAAA365 mutants [underlined amino acid represents an alanine (A) residue replacing an arginine (R) or lysine (K) residue] displayed two affinity states for carbachol. For the AATPA140 and AKAAR365 mutants, agonist high affinity (K_H) and low affinity (K_L) values and the proportion of binding sites present in each affinity state were comparable to those seen for the wild-type receptor (Fig. 2 and Table 1). On the other hand, the KKAAA365 mutant exhibited a wild-type K_L value and a K_H

TABLE 1

Pharmacological parameters for wild-type and arginine/lysine to alanine mutant m1 muscarinic receptor constructs expressed in CHO cells

Receptor construct	[^{3}H]QNB binding		PI hydrolysis, carbachol	
	B_{max}	K_d	EC_{50}	Stimulation
	pmol/mg	*pM*	*μM*	*-fold*
Wild-type	0.82 ± 0.06 (3)	71 ± 2 (3)	5 ± 1 (5)	13 ± 1 (5)
AATPA^{140}	0.52 ± 0.06 (3)	51 ± 13 (3)	4 ± 1 (5)	12 ± 1 (5)
AAAAA^{365}	1.44 ± 0.25 (3)	48 ± 4 (3)	ND (3)	ND (3)
KAAAA^{365}	1.60 ± 0.28 (3)	103 ± 20 (3)	36 ± 0.2[a] (3)	6 ± 1[a] (3)
AKAAA^{365}	0.88 ± 0.19 (4)	60 ± 8 (4)	0.15 ± 0.02[a] (3)	4 ± 1[a] (3)
AAAAR^{365}	0.95 ± 0.21 (3)	82 ± 10 (3)	ND (3)	ND (3)
AKAAR^{365}	0.72 ± 0.15 (3)	58 ± 9 (3)	27 ± 4[a] (3)	13 ± 0.4 (3)
KAAAR^{365}	1.19 ± 0.07 (3)	35 ± 3 (3)	63 ± 7[a] (3)	6 ± 1[a] (3)
KKAAA^{365}	0.64 ± 0.04 (3)	55 ± 7 (3)	5 ± 2 (4)	6 ± 1[a] (4)

ND, not determinable.

Data are mean ± standard error. Values in parentheses represent the number of independent determinations.

[a] Significantly different than corresponding wild-type value ($p < 0.05$).

The basal levels of PI accumulation in transfected cells expressing wild-type, AATPA^{140}, AAAAA^{365}, KAAAA^{365}, AKAAA^{365}, AAAAR^{365}, AKAAR^{365}, KAAAR^{365}, and KKAAA^{365} were, respectively, 1365 ± 191, 1238 ± 188, 1300 ± 178, 1312 ± 146, 1170 ± 328, 1329 ± 142, 1285 ± 125, 1257 ± 263, and 1453 ± 197 dpm.

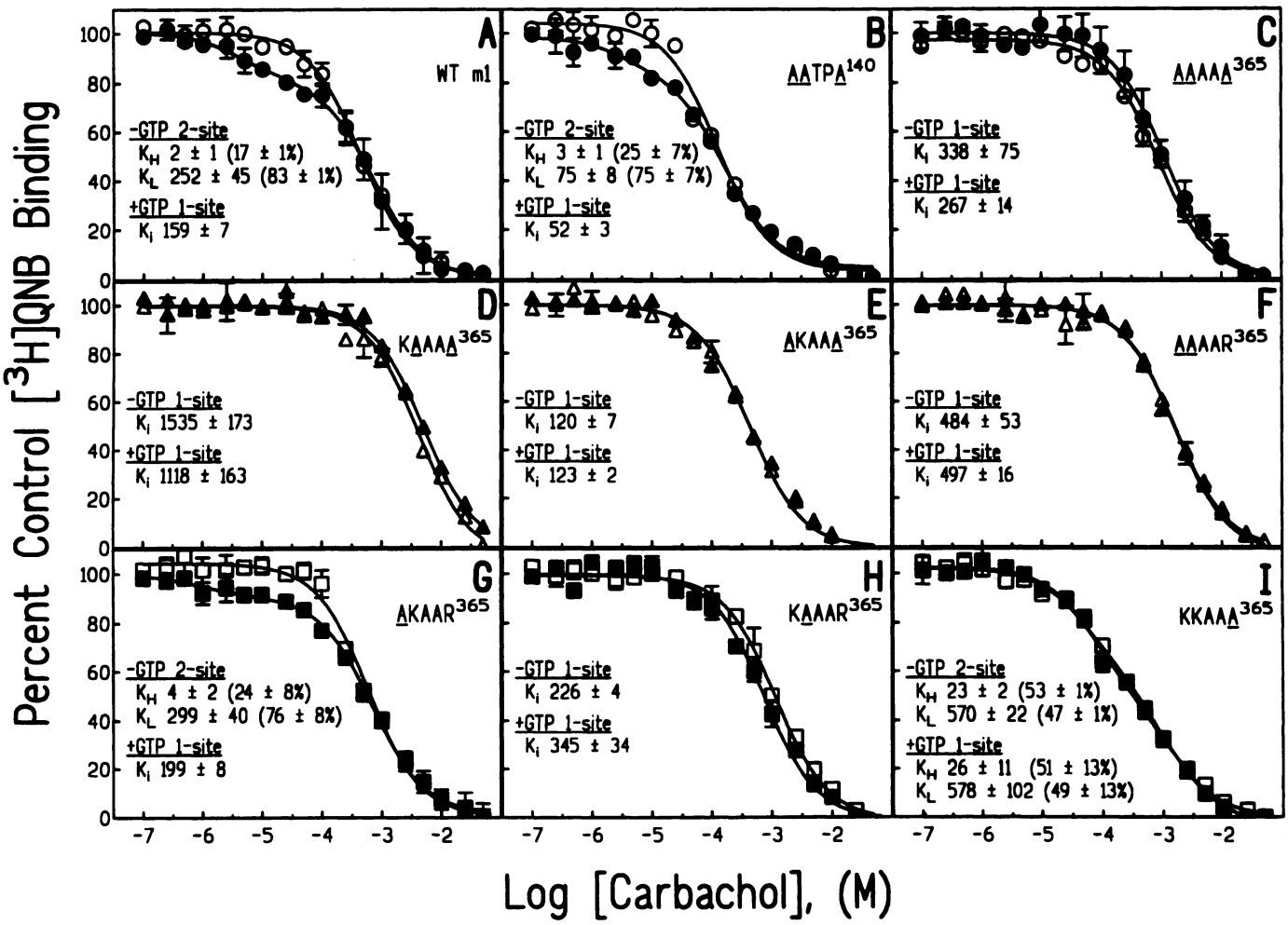

Fig. 2. Carbachol competition binding curves of wild-type and B-B-X-X-B mutant m1 mAChRs. Membranes (15–20 μg of protein) were incubated in 20 mM HEPES, pH 7.4, 100 mM NaCl, and 1 mM $MgCl_2$ for 60 min at 37° in the presence of the indicated concentrations of carbachol (±100 μM GTP) and a concentration of [^{3}H]QNB equivalent to 2 × K_d for each receptor (33). Competition binding experiments were performed in the absence (●, ▲, ■) and presence (○, △, □) of GTP. Curves were best fitted to a one- or two-site model as described previously (31). Agonist affinity values (K_i) are given for curves best fit to a one-site model. K_H (high affinity) and K_L (low affinity) values for carbachol are given for curves best fit to a two-site model. *Values in parentheses*, percentage of total binding sites present in each affinity state. Data represent mean ± standard error of three or four independent experiments performed in triplicate.

value that was significantly higher (~10-fold) than the corresponding wild-type value ($p < 0.05$). The proportion of high affinity sites increased from 17% in the wild-type receptor to 53% in the KKAAA365 mutant. To establish whether the observed redistribution of agonist affinity states is due to partial processing of KKAAA365 mutants into a membrane compartment poorly accessible to carbachol (and other charged molecules, like NMS; see Ref. 37) but not QNB, carbachol/[^{3}H]NMS competition experiments were performed on the KKAAA365 mutant. These experiments demonstrated that the proportion of carbachol high and low affinity sites (K_H = 3 ± 1 μM, 51 ± 3%; K_L = 300 ± 22 μM, 49 ± 3%; two experiments; data not shown) obtained with [^{3}H]NMS as the label was identical to that seen with [^{3}H]QNB (Fig. 2). Therefore, it seems that mutation of the last basic residue affects the relative proportion of high and low affinity states and not agonist accessibility. The remaining mutants exhibited a single low affinity state for carbachol, with agonist affinity values (K_i) in general agreement with the K_L value found on the wild-type receptor. Therefore, the AAAAA365, KAAAR365, KAAAA365, AKAAA365, and AAAAR365 mutants seem to be constrained to an agonist low affinity state. The addition of exogenous GTP to carbachol competition curves eliminates the agonist high affinity binding site in the wild-type, AATPA140, and AKAAR365 receptors while producing no observable changes in the agonist binding parameters of the other mutants (Fig. 2). The significance of the results with GTP are discussed below.

Before further assessing the role of the B-B-X-X-B motif in mAChR coupling, the ability of NaF to stimulate PI hydrolysis was determined to establish that postreceptor PI responses were equivalent in all transfected cell lines used in this study (31). NaF dose-response curves for wild-type and mutant receptors were superimposable with the wild-type receptor exhibiting a maximal level of stimulation 13 ± 1 fold (three experiments) and mutant values ranging from 13 ± 1 to 14 ± 2 fold (two or three experiments) above basal level (data not shown). Basal PI hydrolysis activities for all mutant receptors were not significantly different from wild-type activities, indicating that the constitutive activity of mAChRs in not affected by these basic amino acid mutations (Table 1). Furthermore, the muscarinic antagonist atropine

had no effect on PI hydrolysis in any of the mutants (data not shown). The full agonists carbachol (Table 1) and acetylcholine (data not shown) stimulated PI hydrolysis 13-fold at the wild-type receptor, with an EC_{50} value of 5 ± 1 and 2 ± 1 μM, respectively. In terms of structure-function analysis of m1 mAChR coupling, we first analyzed the effects of mutating the i-2 loop KRTPR140 motif on carbachol-stimulated PI hydrolysis. Carbachol stimulated PI hydrolysis in a wild-type manner at the AATPA140 triple mutant, with an EC_{50} value of 4 μM and a maximal level of stimulation 12-fold above basal (Table 1 and Fig. 3). It is plausible that the i-2 loop B-B-X-X-B motif spans both "activating" and "inactivating" domains. Receptor domains susceptible to activating and inactivating mutations are known to reside next to or to overlap each other (28). Consequently, multiple mutations may have a compensatory effect, thereby masking the functional role of individual basic amino acids in receptor coupling. To account for this possibility, the ARTPR140, KATPR140, and KRTPA140 single mutants were each analyzed for their ability to couple to PI hydrolysis. In each case, however, the single mutants exhibited wild-type coupling, suggesting that the i-2 loop B-B-X-X-B motif is dispensable for agonist activation of PI hydrolysis (data not shown).

A multi-amino acid substitution (KEKKAAR365 to AEAKAAR365) and a deletion mutation (KEKK362 was deleted) of the m1 mAChR (both mutations partially encompass and span beyond the i-3 loop KKAAR365 motif) have been described (25, 26). In these studies, the resulting mutants were wild-type in their ability to couple to PI hydrolysis in CHO cells. Therefore, the exact nature of the KKAAR365 motif in m1 mAChR coupling could not be inferred from these studies. It should be noted that the KEKK362 deletion mutant, although exhibiting wild-type coupling in CHO cells and Rat-2 fibroblasts, did not efficiently couple to PI hydrolysis in Y1 adrenal carcinoma cells (26). A further complication is that the KEKK362 deletion mutation not only deletes the first two basic residues in the KKAAR365 motif but also deletes an acidic residue, Glu360 (26). Mutation of this acidic residue by itself generates an "activated" mutant receptor (28). Thus, we confined our mutations to the KKAAR365 motif to definitively define its role in receptor coupling. In contrast to the results with the i-2 loop AATPA140 triple mutant, carbachol (Fig. 3) and acetylcholine (data not shown) were practically ineffective in stimulating PI hydrolysis at the i-3 loop AAAAA365 triple mutant. This finding, coupled with the inability of the mutant to display GTP-dependent conversion of an agonist high to low affinity state, demonstrates conclusively that the i-3 loop KKAAR365 motif is critical for efficient m1 mAChR coupling (Figs. 2 and 3).

The effects of individually mutating each basic amino acid in the KKAAR365 motif on receptor coupling to PI hydrolysis were assessed (Fig. 4 and Table 1). A previous study has demonstrated that conservative substitutions of Lys361 in the m1 mAChR with serine or the basic residue histidine, amino acids containing electron-donating groups, resulted in mutant receptors exhibiting wild-type efficacy and potency to stimulate PI hydrolysis (26). Our results with the AKAAR365 mutant, which contains a nonpolar alanine substitution at Lys361, suggest that the first basic amino acid in the B-B-X-X-B motif does in fact participate in receptor coupling. Although carbachol maximally stimulated PI hydrolysis at the AKAAR365 mutant in a wild-type manner, the EC_{50} value for this mutant receptor was significantly ($p < 0.05$) increased 5-fold. The reduction in carbachol potency was not associated with a concomitant decrease in either of the two agonist affinity-states of AKAAR365 (compare agonist displacement curves of wild-type and mutant receptors in the absence of GTP, Fig. 2). Therefore, decreases in agonist potency seem to be due to a deficit in the ability of AKAAR365 to couple with G proteins rather than a decrease in agonist affinity to the mutant receptor.

Mutants KAAAR365 and KKAAA365 exhibited striking defects in coupling to PI hydrolysis compared with the AKAAR365 mutant and wild-type receptors (Fig. 4 and Table 1). Maximal stimulation of PI hydrolysis by carbachol declined by 50% at both KAAAR365 and KKAAA365, whereas only at the KAAAR365 mutant did the agonist exhibit a decrease in potency (EC_{50} of carbachol increased ~13-fold in KAAAR365 compared with wild-type). A similar PI hydrolysis coupling defect after a Lys362-to-alanine mutation has

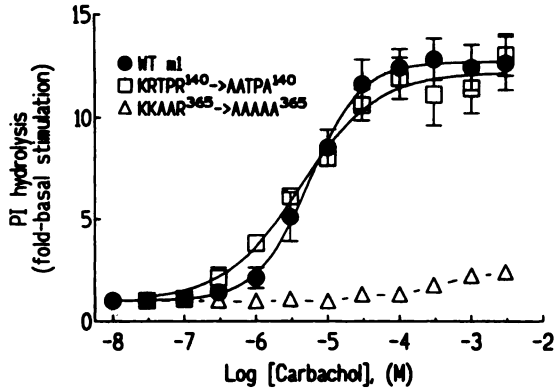

Fig. 3. Dose-response curves for PI hydrolysis mediated by wild-type (*WT*) and triple-substitution B-B-X-X-B mutant m1 mAChRs. Experiments were conducted as described previously (33). Intact cells were stimulated with the indicated concentrations of carbachol for 20 min at 37°. Data are expressed as fold-basal stimulation of PI hydrolysis elicited by carbachol. Basal level of total inositol phosphate release of mutant receptors was not significantly different from wild-type ($p > 0.05$). Data represent mean ± standard error of three to five independent experiments performed in duplicate.

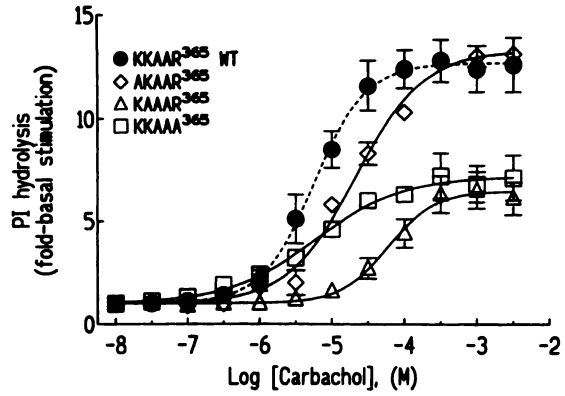

Fig. 4. Dose-response curves for PI hydrolysis mediated by wild-type (*WT*) and single-substitution mutants of the KKAAR365 motif in m1 mAChRs. Experiments were conducted as described previously (33). Data are expressed as fold-basal stimulation of PI hydrolysis elicited by carbachol. Basal level of total inositol phosphate release of mutant receptors was not significantly different from wild-type ($p > 0.05$). Data represent mean ± standard error of three to five independent experiments performed in duplicate.

recently been described (28). Analogous to the AAAAA365 triple mutant, the decreased efficiency of KAAAR365 and KKAAA365 to couple to PI hydrolysis correlated with their inability to display a GTP-dependent conversion of an agonist high affinity state into a low affinity state (Fig. 2). Taken together, our data demonstrate that the first basic amino acid (Lys361) of the B-B-X-X-B motif plays a minor but significant role, whereas the second and third basic residues (Lys362 and Arg365), flanking either side of Ala363 and Ala364, are critical for efficient coupling of m1 mAChRs to PI hydrolysis.

Analysis of the i-3 loop B-B-X-X-B double mutants provided novel insights not discerned with the use of the single mutants regarding the independent and/or hierarchal nature of individual residues facilitating receptor coupling (Fig. 5 and Table 1). Substitution of alanine for Lys361 and Arg365 in the wild-type receptor yielded AKAAA365, a mutant with unusual properties. Although carbachol maximally stimulated PI hydrolysis at AKAAA365 to only ~30% of wild-type levels, the full agonist was 33-fold more potent at this mutant compared with wild-type. A comparison of AKAAA365 with KAAAR365 also reveals that carbachol was 400 times more potent on PI hydrolysis at the former, although the agonist exhibits similar binding properties and elicits a submaximal response at both mutants. It is conceivable that AKAAA365 exhibits an enhanced interaction with its G protein, but the ability to transduce the stimulus response from the mutant to the G protein is impaired. The partial PI response elicited by mutant AKAAA365 in conjunction with data of the single mutants supports the hypothesis that the second basic amino acid Lys362 is both necessary and sufficient for m1 mAChR coupling (Figs. 4 and 5). That is, Lys362 can function independent of surrounding basic amino acids to mediate a partial PI response. Surprisingly, the AAAAR365 mutant was effectively uncoupled from PI hydrolysis, a finding that was not predicted from the results of the single substitution mutants if one assumes that Lys362 and Lys365 function in an additive and independent manner. This finding along with data of the single mutants reveals that the third basic amino acid Lys365 is necessary but not sufficient on its own to facilitate agonist-stimulated PI hydrolysis (Figs. 4 and 5).

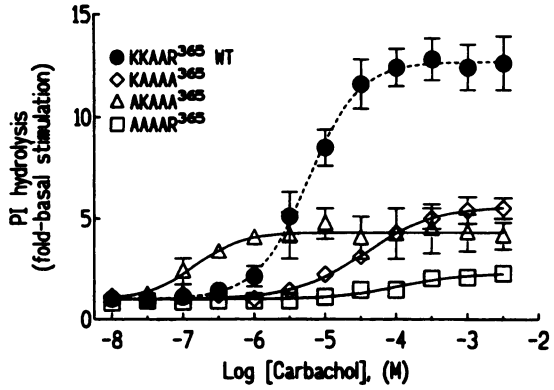

Fig. 5. Dose-response curves for PI hydrolysis mediated by wild-type (*WT*) and double-substitution mutants of the KKAAR365 motif in m1 mAChRs. Experiments were conducted as described previously (33). Data are expressed as fold-basal stimulation of PI hydrolysis elicited by carbachol. Basal level of total inositol phosphate release of mutant receptors was not significantly different from wild-type ($p > 0.05$). Data represent mean ± standard error of three to five independent experiments performed in duplicate.

Apparently, Lys365 requires the presence of either Lys361 or Lys362 in the B-B-X-X-B motif to evoke a partial PI response after receptor activation. As mentioned above, Lys361 is not required for efficient coupling of m1 mAChRs to PI hydrolysis (mutation of this residue affects potency but not maximal stimulation) (Fig. 4). Therefore, simultaneous mutation of the second and third basic amino acids might be expected to yield a mutant receptor, KAAAA365, with little or no ability to couple to PI hydrolysis. Remarkably, PI dose-response curves derived from mutant KAAAA365 reveal that the first basic amino acid can compensate for the absence of the second and third residues. At this particular mutant, carbachol partially activated PI hydrolysis to 50% of wild-type levels (Fig. 5). Thus, strict conformational restraints of the B-B-X-X-B motif seem not to be absolutely obligatory for receptor activation of G proteins.

Of the i-3 loop mutant receptors, the full agonist carbachol was able to maximally activate PI hydrolysis in a wild-type manner only at AKAAR365. In addition, this was the only i-3 loop mutant with the ability to shift from a high to low affinity agonist state in the presence of GTP (Fig. 2). Therefore, the capacity of m1 mAChRs to couple efficiently to PI hydrolysis seems to be correlated with its ability to change conformations, which in turn is dependent on the presence of the second and third basic amino acids in the B-B-X-X-B motif. The ability of full agonists to recognize a high affinity receptor state has been interpreted according to a ternary ligand/receptor/G protein model, in which the high affinity state represents the active form of the receptor coupled to its cognate G protein (38). For mutant α- and β-adrenergic receptors and mAChRs, impairments of G protein activation are generally correlated with an inability of mutant receptors to attain a high affinity agonist state (16, 17, 19, 39–41). In an analogous manner, there exists a strong correlation between the differing abilities of partial muscarinic agonists to activate PI hydrolysis and their ability to recognize the high affinity state of the receptor (42). In the current study, the majority of the mutations introduced into the i-3 loop KKAAR365 motif, with the exception of the Lys361-to-alanine mutation, effectively changed the apparent phenotype of carbachol from a full to partial agonist. Therefore, it was of interest to study the effect of mutations in the i-3 loop KKAAR365 motif on partial agonist function. The partial agonist oxotremorine binds to the mutant receptors in an apparent wild-type manner (Fig. 6A). Oxotremorine/[^{3}H]QNB competition curves reveal a single agonist affinity state for the wild-type receptor (K_i = 0.52 ± 0.02 μM) and the mutants AKAAR365 (K_i = 0.53 ± 0.05 μM), KAAAR365 (K_i = 0.51 ± 0.01 μM), KKAAA365 (K_i = 0.51 ± 0.01 μM), and AAAAA365 (K_i = 0.50 ± 0.01 μM). Likewise, oxotremorine recognized a single affinity state in the i-2 mutant AATPA140 (K_i = 0.53 ± 0.01 μM). For oxotremorine-stimulated PI hydrolysis, all i-3 mutants were functionally uncoupled, whereas the i-2 mutant AATPA140 exhibited activity (EC_{50} = 0.51 ± 0.06 μM; fold-basal stimulation = 5.5 ± 0.7) that was comparable to the wild-type receptor (EC_{50} = 0.42 ± 0.22 μM; fold-basal stimulation = 5.0 ± 0.3) (Fig. 6B). The complete absence of an oxotremorine-stimulated PI response in the AKAAR365 mutant is of particular interest in light of the finding that carbachol elicits a near-wild-type response in this mutant. On the basis of these findings, we propose that the second and third basic amino acids in the i-3 loop B-B-

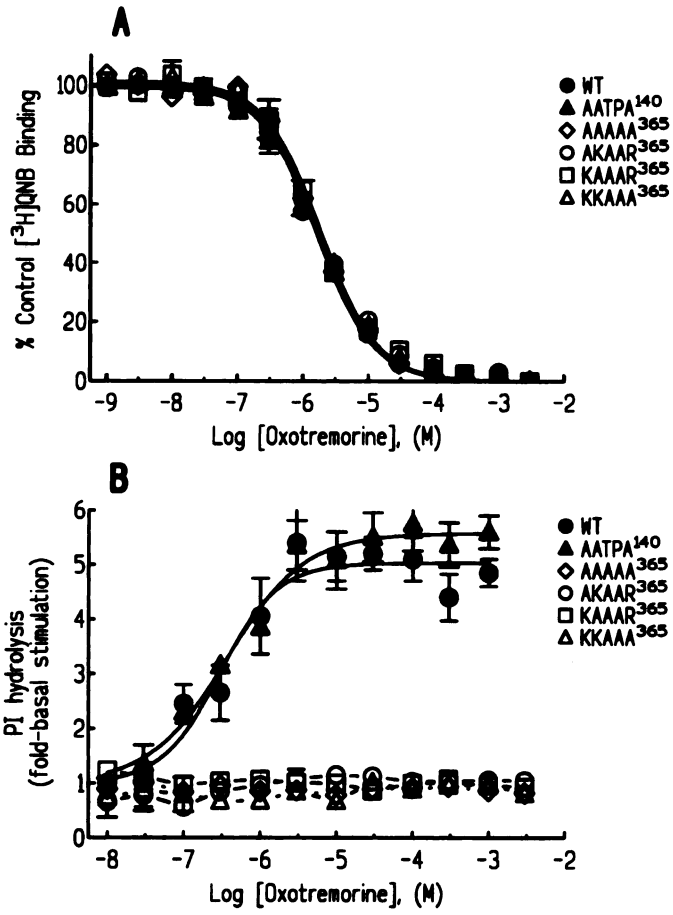

Fig. 6. Oxotremorine binding and activation of wild-type (*WT*) and mutant m1 mAChRs. A, Oxotremorine competition binding curves and data analysis were performed as described in legend to Fig. 2. Data represent mean ± standard error of two or three independent experiments performed in triplicate. B, Oxotremorine dose-response curves for PI hydrolysis and data analysis were performed as described in legend to Fig. 3. Data represent mean ± standard error of two or three independent experiments performed in duplicate.

X-X-B motif are crucial for full agonist recognition of the high affinity agonist state of m1 mAChRs, GTP-dependent conversion of affinity states, and maximal agonist activity. In contrast, all three basic residues must be present for partial agonist activity. Therefore, the ability of partial and full agonists to activate m1 mAChRs seems to be differentially affected by the i-3 mutations.

At equivalent mAChR expression levels in CHO cells, maximal activation of PI hydrolysis by carbachol at m3 mAChRs is 50% of the response elicited from m1 mAChRs (36). It is interesting to note that m3 mAChRs have the motif KKAAQ (where Q is the nonbasic amino acid glutamine) in the carboxyl-terminal i-3 loop, which is in the analogous position as the KKAAR365 motif in m1 mAChRs. Our results with the KKAAA365 m1 mutant, which exhibits half-maximal stimulation of PI hydrolysis compared with wild-type, suggests that the third basic amino acid may account, in part, for the functional differences between m1 and m3 mAChRs. Clearly, future studies are warranted. Previous efforts to define m3 mAChR domains involved in G protein activation have implicated a region near or encompassing the KKAAQ motif in G protein activation (27, 43).

Although results from the AATPA140 mutant imply that

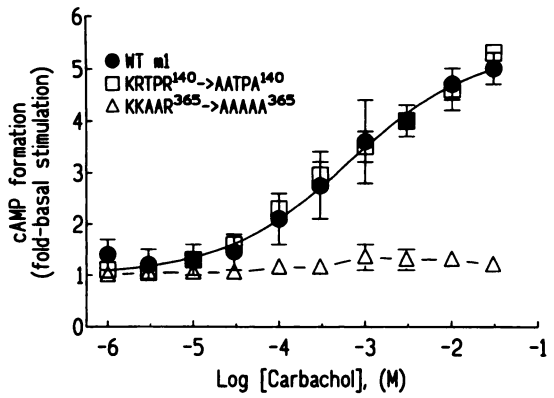

Fig. 7. Dose-response curves for cAMP accumulation mediated by wild-type (*WT*) and triple-substitution B-B-X-X-B mutant m1 mAChRs. Experiments were conducted as described previously (17). Data are expressed as fold-basal stimulation of cAMP accumulation elicited by carbachol. Basal level of cAMP accumulation of mutant receptors was not significantly different from wild-type ($p > 0.05$). Data represent mean ± standard error of two or three independent experiments performed in duplicate.

the i-2 loop B-B-X-X-B sequence was not required for m1 mAChR coupling to PI hydrolysis, it is possible that this motif may be critical for receptor coupling to other signaling pathways. In m1 mAChR-transfected CHO cells, agonist stimulation of cAMP accumulation is independent of the PI hydrolysis pathway (44). Therefore, the i-2 and i-3 loop B-B-X-X-B motifs, the latter being critical for PI hydrolysis, were assessed for their roles in cAMP accumulation. Interestingly, results analogous to those of the PI hydrolysis assays were obtained; carbachol stimulated cAMP accumulation at the i-2 loop triple mutant in a wild-type manner and was completely ineffective at the i-3 loop triple mutant (Fig. 7). These results demonstrate that the i-3 loop B-B-X-X-B sequence may serve as a ubiquitous G protein-activating domain for the purpose of coupling m1 mAChRs to multiple independent signaling pathways. However, it remains to be determined whether this domain participates directly in the activation of other second messenger systems, such as the phospholipase D and mitogen-activated protein kinase pathways (45, 46).

The exact role of the i-2 loop B-B-X-X-B motif remains to be elucidated. This domain is conserved in all five mAChR subtypes in addition to being found in many 7TMD receptors. Interestingly, the i-2 loop B-B-X-X-B motif found in all mAChR subtypes is a consensus sequence for phosphorylation by protein kinase C (where X is the phosphoacceptor amino acid serine or threonine; Ref. 47). That m1 mAChRs are coupled to the $G_{q/11}$/phospholipase C/protein kinase C pathway further supports the premise that this motif may serve an autoregulatory role. Preliminary mutagenesis data from our laboratory suggest that the i-2 loop KRTPR140 sequence participates in agonist-induced receptor desensitization and internalization.[1] Indeed, Haga *et al.* (48) recently demonstrated that a synthetic peptide corresponding to this region is a substrate for phosphorylation by protein kinase C.

In summary, alanine scanning mutagenesis has been used to define the relative roles of each basic amino acid within the G protein-activating motif B-B-X-X-B. Such a motif (KKAAR365) located in the carboxyl-terminal i-3 loop of m1

[1] N. H. Lee, E. Cheng, N. S. M. Geoghagen, and C. M. Fraser, manuscript in preparation.

mAChRs was found to be obligatory for stimulation of both PI hydrolysis and cAMP accumulation in CHO cells, pathways that are independent of each another. In contrast, the KRTPR140 motif in the i-2 loop was dispensable for both second messenger pathways. The second and third basic amino acids of the KKAAR365 motif are required for agonist recognition of a high and low affinity state of the receptor, in addition to being crucial for GTP-dependent conversion of the high affinity state to a low affinity state. Defects in m1 mAChR coupling to second messenger pathways are correlated with the inability of mutants to display these traits. The KKAAR365 motif is necessary (current study) but not sufficient (10, 16, 19, 20, 26, 28) for efficient coupling of m1 mAChRs, supporting the notion that multiple intracellular domains on 7TMD receptors are required for activation of G proteins. Finally, our data provide evidence that individual basic amino acids can function in an independent or interdependent manner for efficacious coupling of m1 mAChRs.

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