Constitutive Activation of the m5 Muscarinic Receptor by a Series of Mutations at the Extracellular End of Transmembrane 6[†]

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ABSTRACT: The m5 muscarinic acetylcholine receptor was constitutively activated by a wide range of amino acid substitutions at a residue (serine 465) that is positioned at the junction of the sixth transmembrane domain and the extracellular loop. Of 13 substitutions tested, 11 produced significant increases in constitutive activity. Replacement of serine 465 with large (phenylalanine and valine) or basic residues (arginine and lysine) increased the constitutive activity of the receptor to between 55 and 110% of the maximum response of the wild-type receptor to the agonist carbachol. Other substitutions (e.g., cysteine and leucine) increased the constitutive activity to an intermediate level (30%), while small and acidic residues (glycine, aspartate, and glutamate) caused small or insignificant increases. The increase in the constitutive activity of each mutant receptor correlated with an increase in the potency of carbachol in both binding and functional assays, with the most constitutively activated receptors showing a 40-fold decrease in the EC_{50} of carbachol. The negative antagonist atropine bound to and reversed the constitutive activity of all mutant receptors with equal potency. These data were fitted to a two-state model of receptor function. The data are consistent with the primary effect of substitutions to serine 465 being to selectively destabilize the inactive state of the receptor, thus favoring formation of the active state in the absence of agonists. Our data strongly support this two-state model of receptor function and identify a critical role of this domain in the activation of muscarinic receptors.

The five muscarinic receptor subtypes (m1−m5, *1*−*4*) are part of the G-protein-coupled receptor family. These receptors have seven transmembrane domains (TM1−7) connected by hydrophilic loops, but little is known about their three-dimensional structure (*5*). Mutagenesis and affinity labeling experiments have placed the ligand binding site of the muscarinic receptor on the transmembrane domains. An aspartate residue in TM3 binds the onium head group of muscarinic ligands (*6*−*8*), and residues where mutations affect agonist but not antagonist binding have been identified in TM2, TM5, TM6, and TM7 (*6*, *9*, *10*, *44*). One residue which appears to selectively affect antagonist binding is located in TM6 (*11*).

Like many other receptors, muscarinic receptors have been shown to activate G-proteins in the absence of added agonists (i.e., they show constitutive activity) (*12*, *13*). This activity can be amplified to measurable levels when receptors or associated G-proteins are overexpressed (*14*−*16*). G-protein-coupled receptors can also be constitutively activated by mutations (*11*, *17*−*20*). In addition to providing insight into the molecular mechanism of receptor activation, these activated G-protein-coupled receptors are of interest due to their association with disease. Retinitus pigmentosa, precocious puberty, and thyroid adenomas have been associated with activating mutations (*21*−*23*).

A simple model that reconciles the ability of receptors to spontaneously activate G-proteins (constitutive activity) with the ability of ligands to both increase and decrease receptor activity is illustrated in Scheme 1 below (*24*, *25*). An extended version of this model which includes terms describing G-protein coupling has also been described (*15*, *26*). In this model (the two-state or allosteric model of receptor activation), R and R* represent inactive and active receptor conformations, respectively. The equilibrium between these conformations is governed by the constant J ($J = [R^*]/[R]$). L represents any ligand, and K and αK represent the dissociation constants governing the interaction between the ligand and R and R*, respectively. Agonist ligands act by binding R* with a higher affinity than R, thus selectively stabilizing R*L. Hence, $\alpha K < K$ for agonist ligands. Two types of antagonists are predicted by this model: neutral antagonists, which bind with equal affinity to R and R* ($\alpha K = K$), and negative antagonists (inverse agonists), which prefer R and thus selectively stabilize RL ($\alpha K > K$). Neutral antagonists would act solely by blocking the effects of agonists, while negative antagonists would both block the effects of agonists and inhibit constitutive activity.

In a previous study, we showed that a muscarinic receptor with two amino acid substitutions at the extracellular end of TM6 had a high level of constitutive activity that was blocked

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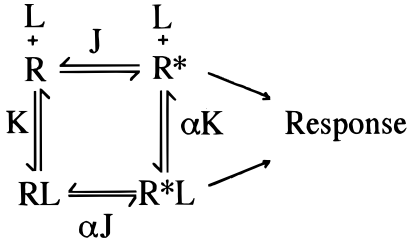
Scheme 1



by atropine and related compounds (*18*). By comparison with other mutant receptors (not shown), we inferred that mutation of serine 465 was responsible for causing this phenotype. To further investigate the structural implications and mechanism causing this phenomenon, we have replaced serine 465 with a series of other amino acids and characterized the phenotypes of the resultant mutant receptors in binding and functional assays.

MATERIALS AND METHODS

Construction of Mutants. Mutant m5 receptors were constructed as described (*27*) in the pCD-m5 plasmid (*3*) by PCR using a primer with the sequence 5′-GCC ATT CTC CTG GCC TTC ATC ATC ACA TGG ACC CCG TAT AAC ATC ATG GTC CTG GTT NNN ACC TTC TGT GAC AAG TGT GTC CC-3′, where N indicates an equimolar mixture of all four bases.

Functional Assays. Receptor selection and amplification technology (R-SAT)[1] assays were used to identify functional receptors and to generate concentration−response curves (*27*−*29*). NIH-3T3 cells are transiently transfected with DNA encoding the receptor and β-galactosidase and exposed to ligands for 4 days. In the absence of receptor activity, NIH-3T3 cells grow to a monolayer only, but in the presence of agonists, cells expressing receptors overcome contact inhibition and proliferate. The assay is stopped while the transfected cells are still in the linear growth phase (*28*). A quantitative measure of cellular proliferation is obtained by measuring the levels of β-galactosidase, a marker enzyme that is constitutively expressed by the transfected cells. R-SAT assays resolve partial agonists from full agonists, and results obtained using this assay compare well with results obtained using inositol triphosphate, GTPase, and whole tissue assays for muscarinic (*28*) and a variety of other receptors (*29*).

R-SAT assays were performed as described (*27*) with minor modifications. DNA was prepared using Qiawell-8 columns and Qiagen gravity flow columns (Qiagen, Chatsworth, CA). NIH-3T3 cells were grown in DMEM supplemented with 10% calf serum unless stated.

Screening. Falcon 24-well plates were seeded with 75 000 cells per well 24 h before transfection with 50 ng of recombinant pCD-m5 plus 150 ng of pSI-β-galactosidase (pSI-β-gal, Promega). After 2 days, the cell culture medium was replaced with DMEM plus 2% cyto-Sf3 (Kemp Laboratories) and either no drug, 100 μM carbachol, or 1 μM atropine. The cells were incubated for 4 days, and β-galactosidase levels were measured as described (*27*). Absorbance measurements were taken at 420 nm. A background absorbance of 0.056 AU (the absorbance of a 96-well plate containing unreacted substrate and no cells) was subtracted from each reading. Using this screening method, cells transfected with wild-type m5 receptors gave approximately 0.15 absorbance unit when incubated with atropine versus 0.9 absorbance unit when incubated with carbachol. All clones giving a difference of more than 0.1 absorbance unit between the carbachol and atropine screens were regarded as functional receptors and were sequenced and examined further in concentration−response experiments.

Concentration−Response Experiments. Three wells of Falcon 6-well plates seeded with 200 000 cells per well were transfected with 0.3 μg of recombinant pCD-m5 and 0.9 μg of pSI-β-gal. Two days after transfection, the cells were divided into 40 wells of 96-well plates containing DMEM plus 2% cyto-Sf3 and 0.5% calf serum. The cells were incubated for 4 days in the presence of no ligand, carbachol dilutions from 30 pM to 100 μM, atropine dilutions from 30 pM to 1 μM, or 10 nM cloprostanol plus 1 μM atropine. β-Galactosidase levels were measured as above. Carbachol EC_{50} values were obtained by fitting the data by nonlinear least-squares analysis (Kaleidograph) to the equation response = minimum + R_{max}[L]/(EC_{50} + [L]). Atropine EC_{50} values were obtained by fitting to the equation response = minimum + $R_{max}EC_{50}$/(EC_{50} + [L]). The maximum receptor response was defined as the difference between the limiting maximum stimulatory response to carbachol (maximum) and the limiting inhibitory response to atropine (minimum).

To account for minor variations in the efficiency of transfections between experiments, R-SAT data were normalized relative to maximum responses to the FP prostenoid receptor that is endogenous to NIH-3T3 cells. The prostenoid agonist cloprostanol (Kayman Chemicals) has an EC_{50} of 1 nM at this receptor, and 10 nM was used to define the maximum response. Atropine (1.0 μM) was also included in these assays to suppress any constitutive activity of the transfected muscarinic receptors.

Radioligand Binding Assays. Six-well plates were seeded with 200 000 NIH-3T3 cells per well and transfected with 0.3 μg of recombinant pCD-m5 and 0.9 μg of pSI-β-gal as described above. Two days after transfection, the cells were harvested, and receptor expression was measured using 1 nM [^{3}H]NMS with or without 1 μM atropine, in triplicate as described (*9*).

TSA cells transfected with 20 μg of DNA per 15 cm plate were used for more detailed studies which were carried out as described (*9*). Data analysis was carried out by nonlinear regression (Kaleidograph). Binding data were initially fitted to the Hill equation [$y = [L]^H/(IC_{50}^H + [L]^H)$, where [L] = the ligand concentration and H = the Hill number], but since antagonist binding data gave curves with a Hill number not significantly different from 1, atropine IC_{50} and [^{3}H]NMS K_d values were recalculated using a Hill number of 1. Atropine IC_{50} values were converted to K_I values according to Cheng and Prusoff (*30*).

RESULTS

We employed a high throughput assay of receptor function (R-SAT, *27*−*29*) to screen a library of recombinant muscarinic receptors where S465 was randomly mutated. As

[1] Abbreviations: EC_{50} and IC_{50}, drug concentrations giving a half-maximal stimulation and inhibition of response, respectively; GppNHp, guanylyl imidodiphosphate; NMS, *N*-methylscopolamine; QNB, quinuclidinyl benzilate; 4-DAMP, 4-(diphenylacetoxy)-*N*-methylpiperidine; oxo-M, oxotremorine-M, respectively; R-SAT, receptor selection and amplification technology, patent pending.

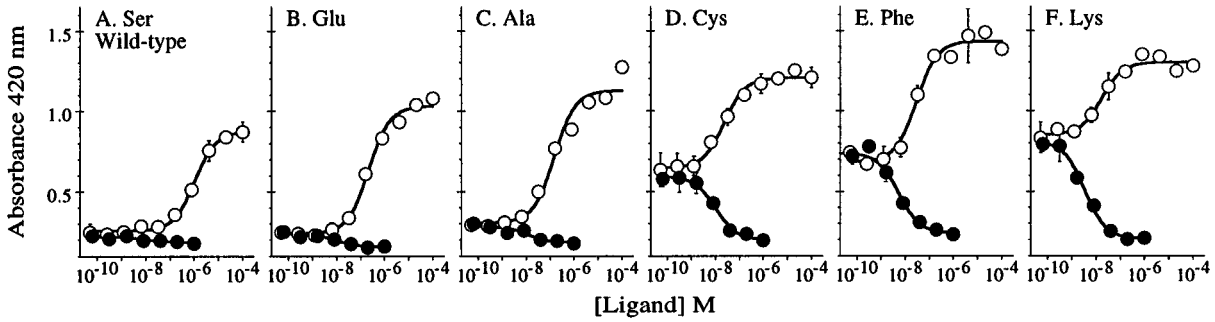


FIGURE 1: Carbachol and atropine concentration−response curves for wild-type and constitutively activated m5 muscarinic receptors. R-SAT functional assays were carried out as described in duplicate using carbachol (○) or atropine (●). (A) Wild-type m5 and (B−F) mutant m5 containing the following amino acids in place of serine 465: glutamate (B, S465E), alanine (C, S465A), cysteine (D, S465C), phenylalanine (E, S465F), and lysine (F, S465K). Points represent the mean ± SEM of data from one representative experiment. Data were not processed in any other way. Solid lines represent the best fit to the equation $y = a + bx/(x + c)$.

Table 1: Functional Data for Recombinant m5 Receptors Mutated at Serine 465[a]

	constitutive activity	maximum receptor response	EC_{50} of atropine (nM)	EC_{50} of carbachol (nM)	fold decrease in the EC_{50} of carbachol
serine	4 ± 2	100 ± 10	not determined	530 ± 170	1.0
aspartate	8 ± 8	130 ± 20	not determined	1200 ± 390	0.44
glycine	8 ± 0	120 ± 20	1 ± 0	220 ± 30	2.4
glutamate	10 ± 1	130 ± 0	9 ± 8	280 ± 60	1.9
proline	17 ± 1	140 ± 6	17 ± 6	350 ± 60	1.5
tryptophan	18 ± 1	150 ± 20	6 ± 4	110 ± 6	5.0
alanine	20 ± 0	160 ± 1	5 ± 2	150 ± 2	3.5
leucine	29 ± 3	140 ± 8	8 ± 2	28 ± 1	19
cysteine	29 ± 3	140 ± 1	5 ± 3	51 ± 6	10
valine	56 ± 3	180 ± 4	3 ± 0	36 ± 4	15
phenylalanine	69 ± 6	170 ± 7	4 ± 2	27 ± 1	20
arginine	77 ± 5	160 ± 3	8 ± 1	18 ± 1	29
tyrosine-pro[b]	90 ± 16	160 ± 20	4 ± 1	13 ± 2	40
lysine	110 ± 6	180 ± 1	6 ± 2	15 ± 2	36

[a] Data were obtained using R-SAT assays. Values represent the mean ± SEM of two independent transfections. Constitutive activity is defined as the atropine-inhibitable response in the absence of agonists. Maximum receptor response is defined as the sum of the maximum response to carbachol and the negative response to atropine. Response values were normalized to an internal standard and then expressed as a percentage of the wild-type maximum response. [b] Tyrosine-Pro indicates a double mutant with the mutations serine 465 to tyrosine and threonine 466 to proline, described in ref 18. No atropine EC_{50} is given for the wild type (serine) or the aspartate mutant because the low basal responses of these receptors made it impossible to determine this value.

shown in Figure 1A, a robust, concentration-dependant increase in β-galactosidase levels can be observed when NIH-3T3 cells cotransfected with DNA encoding the genes for m5 receptors and β-galactosidase are incubated in the presence of carbachol for 4 days. Cells transfected with wild-type receptors (wild-type Figure 1A) produced a basal response of 0.2 AU when incubated in the absence of ligands. Exposure to the agonist carbachol increased this response by 0.75 AU, while the negative antagonist atropine had no significant effect on the measured response.

Figure 1 also shows unprocessed concentration−response data from five representative experiments carried out in parallel using mutant receptors where S465 is replaced with acidic, small neutral, hydrophobic, and basic residues. Considering the highly constitutively activated mutant receptor S465F (where serine 465 is mutated to phenylalanine, Figure 1E), it can be seen that cells expressing this mutant showed a very high basal response in the absence of agonists (0.7 AU for S456F versus 0.2 AU for the wild type). This increased basal activity was suppressed by atropine to a level which was not significantly different from the basal response of the wild-type receptor. The atropine-inhibitable response of the receptors was 0.6 AU for S465F versus 0.02 AU for the wild type. The receptor response was further stimulated by carbachol, which was 20-fold more potent on the S465F mutant than on wild-type m5 (EC_{50} of carbachol = 30 nM for S465F versus 1 μM for the wild type), and produced a greater maximum response (maximum receptor response = 1.2 AU for S465F versus 0.75 AU for the wild type).

Table 1 summarizes functional data obtained from 12 receptors where S465 was replaced by a wide variety of amino acids. Data from the double mutant S465Y/T466P are also included (*18*). Data are arranged in the order of increasing constitutive activity. Mutation of S465 to aspartate, glycine, and glutamate produced little or no change either in constitutive activity of the receptor or in the EC_{50} of carbachol. Other mutations caused varying levels of constitutive activity, with mutations to phenylalanine, arginine, and lysine causing much higher constitutive activity levels than mutations to alanine, leucine, and cysteine. In every case, the observed constitutive activity was completely suppressed by atropine. In general, increases in constitutive activity were associated with increases in the maximum response. No large or systematic variations were seen in the potency of atropine for the constitutively activated receptors, but increases in the potency of carbachol were seen for all mutants except S465D. As illustrated in Figure 2, a strong correlation existed between the observed increases in carbachol potency and constitutive activity.

Radioligand binding assays carried out in parallel on equivalent plates of NIH-3T3 cells transfected with either wild-type receptors or the highly constitutively activated

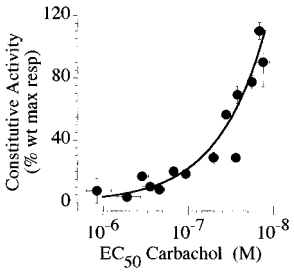


FIGURE 2: Correlation of carbachol potency with the constitutive activity level observed for each mutant receptor. Comparison of the EC_{50} values of carbachol derived from functional studies versus the constitutive activity level of the mutant receptors. Points represent the mean constitutive activity level and EC_{50} values of carbachol as shown in Table 1 and ref 18. Solid line represents the best fit of these data to the logistic equation $y = ax^b$.

mutant S465Y/T466P showed that these receptors were expressed at equal levels [wild type, 2.6 ± 0.7 fmol/(well of a 6-well plate); S465Y/T466P, 2.5 ± 0.9 fmol/(well of a 6-well plate)]. Each well contained sufficient cells for 20 assay points in an R-SAT assay. Because only a minority of cells used in an R-SAT assay express receptors, the receptor number per cell cannot be measured; however, these receptors are expressed at equal levels by the overall population of transfected cells.

More detailed radioligand binding studies were carried out on six representative mutant receptors, including receptors with low, intermediate, and high levels of constitutive activity expressed in TSA cells. No systematic differences were seen in the expression levels of the recombinant receptors (picomoles per milligram of protein, mean ± SEM; wild type, 34 ± 16; S465G, 44 ± 37; S465A, 19 ± 12; S465V, 17 ± 8; S465R, 33 ± 2; and S465F, 11 ± 6). The antagonists [^{3}H]NMS and atropine bound all six receptors with similar affinities (Table 2, Figure 3A); however, the agonist carbachol was up to 50-fold more potent on the constitutively activated receptors than on the wild type (Figure 3B). The IC_{50} of carbachol measured in radioligand binding studies correlated well with the EC_{50} of carbachol observed in functional assays (Figure 4). Carbachol produced shallow binding curves (Hill number ≈ 0.8) on both the wild-type and constitutively activated m5 receptors despite the inclusion of 50 μM GppNHp in these assays. There was no significant difference in the Hill numbers of the curves for the wild-type-like and constitutively activated receptors.

DISCUSSION

Substitution of serine 465 (S465) with a wide range of amino acids constitutively activates the m5 muscarinic receptor (Figure 1, Table 1). Of 13 mutants tested, 11 showed significant increases in constitutive activity, with substitutions as diverse as cysteine, arginine, and phenylalanine producing receptors with this phenotype. These residues are unlikely to make similar interactions within the receptor, and we therefore suggest that mutation of S465 disrupts interactions which hold the receptor in an inactive conformation. Wild-type receptors exhibit low levels of constitutive activity (under 5% of the maximum response to carbachol), and thus, although a fraction of the receptor must be present in an active form (R*), at least 95% of the unoccupied, wild-type receptor must normally be present in an inactive conformation (R). Mutation of S465 may selectively destabilize this inactive receptor conformation so that in the absence of ligands as little as 50% of the receptors are in the inactive conformation and up to 50% are in the active, R* conformation. A similar argument has been used to explain the constitutive activity of a series of adrenergic receptor mutants (*17*).

It is unlikely that the hydroxyl group of S465 is directly involved in any constraining interaction or in any critical posttranslational modification, hydrogen bond, or other internal protein interaction, since these would be destroyed by mutation of S465 to glycine, alanine, or proline, and these substitutions produced a receptor phenotype very close to the wild type. The hydroxl group of S465 is also unlikely to be directly involved in the activation mechanism of the receptor, since the mutant receptors remained fully functional.

Large and basic residues at position 465 cause the highest levels of constitutive activity, while small and acidic residues cause lower levels. This pattern suggests that S465 may occupy a spatially restricted position in the inactive R conformation but in a more open position the active R* conformation. Figure 6 shows two possible mechanisms of receptor activation which could produce this effect. In the R conformation, S465 may face toward another part of the receptor (e.g., another helix, as illustrated in Figure 6A). On activation by an agonist, TM6 may physically move relative to this domain such that S465 now faces toward the extracellular space, its interface with the membrane, or a noncritical part of the receptor protein. In this way, mutation of S465 would destabilize the R conformation by weakening interactions between TM6 and the other domain, but the R* conformation would be unaffected. Recent results on rhodopsin suggest a similar activation mechanism (*31, 32*).

This hypothesis is compelling for several reasons. It explains why different residues cause different levels of constitutive activity, because the interaction between TM6 and the other domain would be destabilized to different degrees depending the size and charge of the substituted residue. It also explains why hydrophobic and basic substitutions can produce the same phenotype, because neither residue type might be tolerated in the confined space between TM6 and the other domain. Finally, since no interactions in either the R or the R* conformation are destroyed (they are only weakened), the receptor would remain fully able to assume both conformations and its abilities to bind agonists and antagonists and activate G-proteins would remain intact.

Mutation of S465 has little effect on atropine binding by the receptor. Atropine reversed the constitutive activity of all 12 mutants tested and acted with an EC_{50} of between 3 and 9 nM on 10. In radioligand binding assays, [^{3}H]NMS and atropine bound with almost equal affinity to the wild-type receptor and five mutants having low, intermediate, and high constitutive activity. S465 thus appears to make no direct interaction with atropine or NMS. More detailed studies (*16, 18*) have shown that five antagonists (atropine, pirenzepine, 4-DAMP, QNB, and NMS) act with similar potencies on the wild-type receptor and the highly constitutively activated mutant S465Y/T466P and completely suppress the constitutive activity of both. Mutation of S465 thus appears to have no effect on the binding sites of these antagonists. These data suggest that the overall architecture of the inactive, R conformation is probably not disrupted by mutation of S465 because this would be expected to affect the receptor's affinity for these negative antagonists.

Table 2: Radioligand Binding Properties of m5 Receptors Mutated at Serine 465[a]

	[^{3}H]NMS		atropine		carbachol		
residue	K_D (nM)	fold change	K_I (nM)	fold change	IC_{50}	Hill	fold change
serine	0.48 ± 0.27	1.0	1.8 ± 0.6	1.0	32 ± 8	0.86 ± 0.04	1.0
glycine	1.0 ± 0.6	0.5	2.5 ± 2.0	0.7	14 ± 6	0.82 ± 0.15	2.3
alanine	0.31 ± 0.02	1.5	0.97 ± 0.50	1.9	13 ± 1	0.74 ± 0.04	2.5
valine	0.31 ± 0.11	1.5	0.73 ± 0.37	2.5	2.4 ± 0.4	0.79 ± 0.01	13
arginine	1.5 ± 0.8	0.3	1.6 ± 0.7	1.2	0.93 ± 0.20	0.79 ± 0.02	35
phenylalanine	0.44 ± 0.16	1.1	1.1 ± 0.45	1.6	0.66 ± 0.15	0.78 ± 0.00	49

[a] Binding assays were carried out in the presence of 50 μM GppNHp. Competition assays were carried out using 360pM [^{3}H]NMS. Saturation assays were carried out using six concentrations of [^{3}H]NMS. Data were analyzed by nonlinear regression (see Materials and Methods). Atropine and NMS binding curves gave Hill numbers not significantly different from 1. K_I values were calculated for atropine using the Cheng-Prusoff equation. Values represent the mean of two independent transfections.

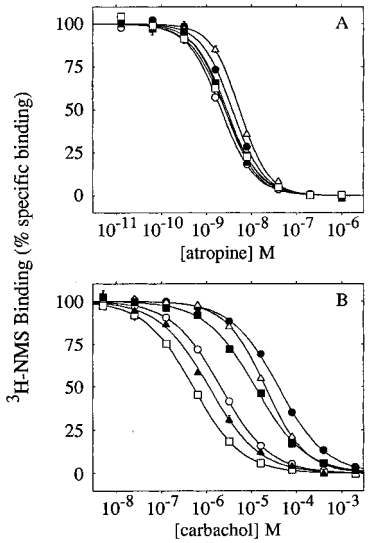


FIGURE 3: Radioligand binding assays for m5 receptors mutated at serine 465. Atropine (A) and carbachol (B) were used to inhibit the binding of 360 pM [^{3}H]NMS to membranes prepared from TSA cells expressing the following receptors: (●) wild type, (△) glycine, (■) alanine, (○) valine, (▲) arginine, and (□) phenylalanine. Assays were carried out in 25 mM phosphate buffer (pH 7.5), 5 mM $MgCl_2$, and 50 μM GppNHp and were equilibrated at 25 °C for 4 h as described (*9*). Points represent the means of data from a representative experiment carried out twice in duplicate. All curves were fitted to the equation $y = IC_{50}^H/(IC_{50}^H + [L]^H)$, where [L] = the ligand concentration and H = the Hill number. The fitted parameters are summarized in Table 2.

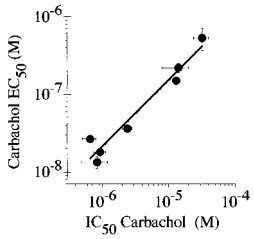


FIGURE 4: Comparison of the EC_{50} values of carbachol derived from functional assays with the IC_{50} value of carbachol derived from radioligand binding assays. Data relating to seven mutant receptors (including mutants of high, intermediate, and low constitutive activity) and the wild-type receptor are shown. Points show EC_{50} and IC_{50} values (±SEM) taken from Tables 1 and 2 and ref 18. The line shows the best fit of the data to a straight line.

In contrast to the results with atropine, 12 out of 13 amino acid substitutions tested produced decreases of up to 50-fold in the EC_{50} of carbachol measured in functional assays. Since almost identical shifts in the IC_{50} of carbachol were observed in radioligand binding assays, this probably reflects an increase in the affinity of the mutant receptors for

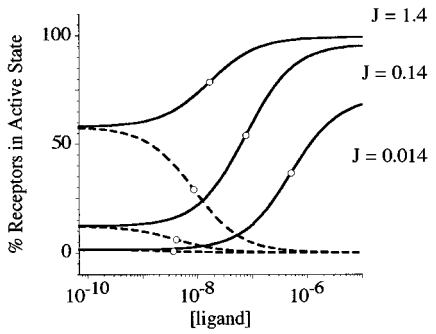


FIGURE 5: Predicted effect of increases in J on the constitutive activity and the potency of agonists and antagonists on a mutant receptor. Simulated data was calculated according to the model of receptor activation shown in Scheme 1 and solved in eq 1. % receptors in the active state is defined as $([R^*] + [R^*L])/R_T$. Solid lines show the predicted effect of adding an agonist ($-\log K = 5.5$ and $-\log \alpha = 2.5$). Dotted lines show the predicted effect of a negative antagonist ($-\log K = 8.5$ and $-\log \alpha < -2.5$).

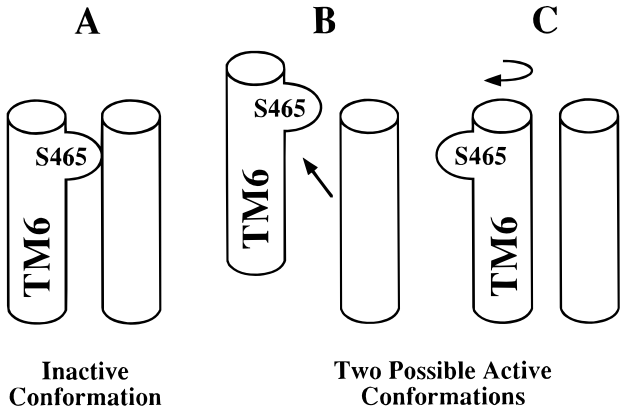


FIGURE 6: S465 may be positioned in a constrained environment in the inactive state and a constrained environment in the active state. In the inactive receptor conformation (A), we suggest that S465 may be positioned at the interface between TM6 and another protein domain. On activation by agonists, we suggest that TM6 physically moves relative to this interacting domain, for example by displacement (B) or rotation (C). This movement may shift the position of S465 so that it lies in a less restricted or otherwise noncritical environment. In this way, mutations to S465 can destabilize the inactive receptor conformation (R) without affecting the active conformation (R*).

carbachol. Two lines of evidence suggest that these increases in carbachol potency are a direct consequence of the increased constitutive activity of the mutant receptors. As shown in Figure 5, mutations which increase constitutive activity by stabilizing R^* relative to R (increasing J) are predicted to cause increases in agonist potency (*24*, *26*). Firstly, a strong correlation exists between the increases in carbachol potency and constitutive activity (Figure 2),

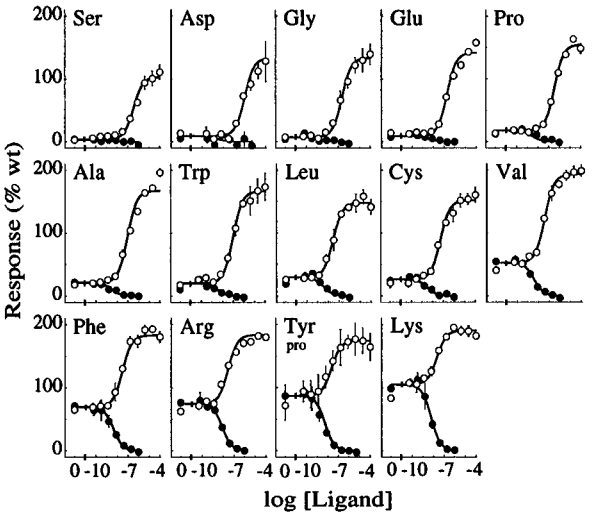


FIGURE 7: Analysis of carbachol and atropine concentration−response curves for m5 muscarinic receptors mutated at serine 465 according to the two-state model of receptor activation. R-SAT functional assays were carried out as described in duplicate using carbachol (○) or atropine (●). Response levels were normalized using an internal standard as described in Materials and Methods and are expressed relative to the maximum receptor response obtained from the wild-type receptor. Points represent the mean ±SEM of data from two to four experiments. 0 represents no drug on the *x*-axis. Solid lines represent the best fit of the data to the equation response = $AR_TJ(1 + [L]/\alpha K)/(1 + J + [L]/K + J[L]/\alpha K)$. Curve fitting and parameters are summarized in the Appendix and Table 3.

strongly suggesting that both effects are caused by a single phenomenon. This finding is in contrast to the results of Kjelsberg et al. (*17*), who found no correlation between agonist potency and constitutive activity levels in a series of mutant adrenergic receptors. Secondly, as described in ref 18, mutation of S465 also increases the potency and affinity of agonists having structures very different from carbachol (oxotremorine-M, arecoline, pilocarpine, and McN-A-343), by an amount proportional to the efficacy of the agonist tested, as predicted by the model in Scheme 1 (*24*). This would not be expected if the observed increases in the potency of carbachol were caused by a specific change in the carbachol binding site. The hydroxyl group of S465 is unlikely to interact with carbachol because its removal (e.g., in the alanine and proline mutants) causes little change in the affinity of the receptor for carbachol.

Because mutations of S465 increase constitutive activity by 30-fold while receptor expression is unchanged, these mutations probably affect the equilibrium between the inactive and active conformations of the unliganded receptor (governed by the parameter *J* in Scheme 1). To determine whether the mutations also affected the affinity of R and R* for carbachol (i.e., K_{CCh} and α_{CCh}) or the maximum response obtainable from the receptor (AR_T), we simultaneously fitted the functional data obtained with these mutants to the model shown in Scheme 1. In the design of this fitting procedure (which is described in the Appendix), it was necessary to assume that some parameters were equal for several receptors; however, the validity of this assumption was tested independently for each parameter on each mutant and individually assigned parameters were used where necessary. Figure 7 shows the results of this curve fitting exercise, and this mechanistic model fits the data extremely well. Table 3 lists the parameter values obtained. Mutations of S465 caused increases of up to 60-fold in the value of *J*, while AR_T, K_{CCh}, and α_{CCh} remained largely unaffected. The observed increases in carbachol potency are therefore probably caused primarily by increases in *J*. There is no evidence that mutation of serine 465 directly causes an increase in the affinity of either the R or R* receptor conformation for carbachol, and it is extremely unlikely that S465 itself makes any direct contact with carbachol. The implication of this result is that mutation of S465 appears to have no major effect on either the R or R* receptor conformations, but simply affects the equilibrium existing between the two states.

Using this model, we have shown that both the constitutive activity and the increased potency of carbachol for these mutant receptors can be explained as a consequence of increasing the relative stability of the active, R* receptor conformation. However, the model provides no explanation for the shallow radioligand binding curves obtained using carbachol. Shallow agonist binding curves (with Hill numbers of less than 1) are commonly seen with muscarinic receptors in both membrane and purified preparations, even in the presence of GppNHp (*18*, *33*−*34*). This phenomenon has been variously attributed to cooperative interactions associated with oligomeric receptor structures and receptor−G-protein complexes (*33*−*36*). Whatever the explanation for shallow carbachol binding curves, the wild-type and mutant receptors were similar in this property, having Hill numbers of approximately 0.8, indicating that this phenomenon is unrelated to the degree of constitutive activity and overall carbachol potency.

Although a striking correlation existed between the potency of carbachol for the mutant receptors as measured in functional assays (EC_{50}) and binding assays (IC_{50}, Figure 4), carbachol was consistently around 30-fold less potent in the binding assays. This phenomenon is commonly seen in both tissue (*37*, *38*) and cell culture (*27*) preparations and has been attributed to the cellular response becoming saturated when only a proportion of the receptors are occupied by agonist (i.e., there are "spare receptors"; *37*, *38*).

Serine 465 is a nonconserved residue and probably lies at the extreme extracellular end of TM6 (*3*, *39*). Charged residues are tolerated at position 465; therefore, this residue is unlikely to face into the lipid of the membrane. Proline and glycine are also tolerated (and give a phenotype very close to the wild type), and since these residues rarely occur within the body of α-helices, S465 is more likely to function as a "capping residue" for TM6 (*40*).

Mutations of S465 at the extracellular end of TM6 appear to cause receptor activation by disrupting the inactive receptor conformation through unfavorable charge or steric effects. An implication of this is that ligands which bind to S465 could potentially have the same effect, and would therefore act as agonists. These ligands could potentially bind the receptor simultaneously with carbachol (which does not appear to bind S465) and might increase its affinity for the receptor (as alcuronium increases the affinity of the receptor for the antagonist NMS; *41*). Because carbachol [$NH_2COOCH_2CH_2N^+(CH_3)_3$] and acetylcholine [$CH_3COOCH_2CH_2N^+(CH_3)_3$] are generally presumed to act through the same pharmacophore (e.g., *42*), these ligands could be used to potentiate the binding of endogenous acetylcholine in an acetylcholine-deficient brain. This would have implications for the treatment of Alzheimer's disease. Additionally, because the acetylcholine binding site of the

Table 3: Analysis of Functional Response Data According to the Two-State Model of Receptor Activation[a]

	log J	J	AR_T	$-\log K_{CCh}$	$-\log \alpha_{CCh}$	$-\log K_{Atr}$
SHARED VALUE			188 ± 3	6.13 ± 0.05	1.80 ± 0.06	8.55 ± 0.06
serine	-1.72 ± 0.05	0.02	SV[b]	SV	SV	SV
glycine	-1.41 ± 0.05	0.04	SV	SV	SV	SV
glutamate	-1.31 ± 0.04	0.05	SV	SV	SV	SV
aspartate	-1.25 ± 0.17	0.06	171 ± 16	5.34 ± 0.17	SV	SV
proline	-0.99 ± 0.06	0.10	SV	5.74 ± 0.12	1.65 ± 0.09	SV
tryptophan	-0.92 ± 0.04	0.12	SV	SV	SV	SV
alanine	-0.90 ± 0.04	0.13	SV	SV	SV	SV
cysteine	-0.71 ± 0.05	0.20	161 ± 5	SV	1.91 ± 0.11	SV
leucine[c]	$-0.62^c \pm 0.05$	0.24^c	$153^c \pm 4$	6.13^c	$2.12^c \pm 0.10$	SV
valine[c]	$-0.45^c \pm 0.03$	0.35^c	$200^c \pm 3$	6.13^c	$1.87^c \pm 0.07$	SV
phenylalanine	-0.23 ± 0.02	0.59	SV	SV	SV	SV
arginine	-0.18 ± 0.02	0.68	SV	SV	SV	8.30 ± 0.13
tyrosine-pro	0.02 ± 0.02	1.05	175 ± 25	SV	SV	8.81 ± 0.11
lysine	0.08 ± 0.03	1.19	193 ± 3	SV	SV	SV

[a] Fitted curves obtained using these parameters are shown in Figure 7. Data from between two and four independent R-SAT concentration−response experiments were simultaneously fitted to the two-state model (shown in Scheme 1) as described in the Appendix. [b] SV indicates that the value of this parameter is not significantly different from the shared value shown on line 1 of the table. Values for parameters which were determined to be significantly different from the shared value are provided as figures. [c] For the valine and leucine mutants, it was neccessary to fix one parameter value (see the Appendix). The value of $-\log K_{CCh}$ was fixed at 6.13 to allow values for the other parameters to be estimated.

muscarinic receptors is necessarily conserved between the five receptor subtypes while the extracellular loops are not, agonists which bind to S465 (itself an unconserved residue) potentially have far greater selectivity than conventional agonists. We therefore suggest that ligands acting through serine 465 have great potential as pharmaceuticals, and that this series of mutant receptors could be used to identify these ligands.

APPENDIX

Simultaneous Analysis of Functional Data

Scheme 1 illustrates the two-state (allosteric) model of receptor activation (*24*). To fit data to this model we made the following assumptions. (1) Total receptor concentration $R_T = [R] + [RL] + [R^*] + [R^*L]$. (2) R* and R*L elicit equal responses from the cell. (3) The cellular response is proportional to the sum concentration of R* and R*L; hence, response $= A([R^*] + [R^*L])$.

Using these assumptions, the two-state model can be solved to give the following expression:

$$\text{response} = AR_T \frac{J(1 + [L]/\alpha K)}{1 + [L]/K + J(1 + [L]/\alpha K)} \quad (1)$$

Since eq 1 contains four parameters (AR_T, J, K, and α), and a concentration−response curve defines only three (basal activity, maximum response, and EC_{50}), it is impossible to solve each curve independently. However, if it can be shown that some parameters are shared by more than one mutant, it is possible to simultaneously fit the entire data set.

The entire data set shown in Figure 7 was fitted to eq 1 using nonlinear least-squares regression as described in ref 33. All parameters except AR_T were defined as logarithms during the fitting procedure. $-\log \alpha_{Atr}$ was assumed to be -4. This was justified because atropine suppressed the constitutive activity of all receptors tested to a level indistinguishable from the basal response of the wild-type receptor. A very acceptable fit was obtained when K_{Atr}, K_{CCh}, α_{CCh}, and AR_T were held equal for all 14 receptors but separate values of J were defined for each receptor. In contrast, the data could not be acceptably fitted when J, K_{Atr}, K_{CCh}, and α_{CCh} were held equal for all 14 receptors and AR_T was defined separately, or when J and AR_T were held equal for all 14 receptors and K_{CCh} and α_{CCh} were defined separately. We concluded that J must be separately defined for each mutant to fit the data.

To improve the curve fit obtained using shared values of K_{Atr}, K_{CCh}, α_{CCh}, and AR_T for all 14 receptors, a statistical method was used to identify mutants where these parameters differed from the shared value. Each parameter was individually released for each mutant, and the data set was fitted again. Parameters were defined as being different from the shared value if a significant improvement was seen in the goodness of fit (as defined by the F statistic, *43*). For example, when the data set was fitted with a separate value of AR_T defined for S465K but AR_T was held equal for the other 13 receptors, there was a significant improvement in the goodness of fit compared to when AR_T was held equal for all 14 receptors ($F = 4.0$, $p < 0.05$). Thus, AR_T for S465K was defined as being different from the shared value of AR_T. In contrast, there was no improvement in the goodness of fit when K_{CCh} for S465K was defined separately ($F = 1.8$, $p > 0.05$), and thus, K_{CCh} for S465K was defined as being not significantly different from the shared value of K_{CCh}.

The entire data set was then fitted again with single (shared) values of AR_T, K_{CCh}, and α_{CCh} assigned in all cases where separately defining these parameters made no difference to the curve fitting (e.g., K_{CCh} for S465K, indicated SV in Table 3), and separate values of AR_T, K_{CCh}, and α_{CCh} were assigned where a significant difference was observed (e.g., AR_T for S465K). The curve fitting procedure was repeated to determine the value of K_{Atr} for the mutants.

For the mutants S465V and S465L, significant improvements in the curve fitting were obtained by releasing any one of J, AR_T, K_{CCh}, and α_{CCh}. Since the concentration−response curves cannot be fitted to eq 1 without at least one shared parameter, the values of these parameters were undefined in the final analysis; i.e., no significant decrease in the sum of squares was observed with values of $\log K_{CCh}$ between 0 and 7, $\log \alpha_{CCh}$ between 1 and 8, or AR_T between 148 and 350 (S465L) or 193 and 350 (S465V). We therefore fixed the value of K_{CCh} for these mutants at 6.13, to determine

whether the remaining parameters would give values close to those defined for the other mutants.

For the mutant S465Y/T466P, different values of AR_T were obtained for the two data sets which were used in this curve fitting procedure. Separate values of AR_T were therefore defined during the final curve fitting procedure, and the mean of these two values is presented in Table 3.

The results of the final curve fitting exercise are illustrated in Figure 7 and summarized in Table 3. Since a very satisfactory fit of the data was obtained when the values of AR_T, K_{Atr}, K_{CCh}, and α_{CCh} were constrained to be equal for all 14 mutants, it was not surprising that in 70% of cases (indicated SV in Table 3) no improvement was observed in the fitted curves when these parameters were independently defined for the individual mutants. Where deviations from these shared values were noted, these were less than 0.4 log unit in 96% of the cases (with one exception, K_{CCh} for S465D). In contrast, the value of J varied by 60-fold across the data set. Hence, the primary effect of mutations in S465 appears to be in increasing the value of J.

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