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Assessment of Mechanistic Proposals for the Binding of Agonists to Cardiac Muscarinic Receptors†

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ABSTRACT: *N*-[^{3}H]Methylscopolamine has been used to characterize muscarinic receptors in crude homogenates prepared from hearts of Syrian golden hamsters. The Hill coefficient is one for specific binding of the radioligand itself and for its inhibition by muscarinic antagonists; markedly lower values are obtained for its inhibition by muscarinic agonists. The binding patterns of agonists have been analyzed in terms of a mixture of sites differing in affinity for the drug and reveal the following. (1) All agonists discern at least two classes of receptor in atrial and ventricular homogenates. (2) The number of classes and the relative size of each differ for different agonists in the same region and for the same agonist in different regions. (3) Atrial and ventricular affinities are in good agreement for some agonists but differ for others. (4) Guanylyl imidodiphosphate (GMP-PNP) is without effect on the specific binding of the radioligand but alters the binding of carbachol via an apparent redistribution of receptors from one class to another; the apparent affinity at either class remains unchanged. (5) Carbachol reveals two classes of sites in ventricular preparations, and the nucleotide mediates an interconversion from higher to lower affinity; three classes are revealed in atrial preparations, and the nucleotide eliminates the sites of highest affinity with a concomitant increase in the number of sites of lowest affinity. Taken together, the data are incompatible with the notion of different, noninterconverting sites; rather, there appear to be several possible states of affinity such that the equilibrium distribution of receptors among the various states is determined by the tissue, by the agonist, and by neurohumoral modulators such as guanylyl nucleotides. The effects of agonists and GMP-PNP cannot be rationalized in terms of a ternary complex model in which the low Hill coefficients arise from a spontaneous equilibrium between receptor (R) and G protein (G) and in which agonists bind preferentially to the RG complex.

Hill coefficients for the specific binding of drugs in equilibrium with membrane-bound, muscarinic receptors are near or equal to 1 for antagonists and significantly lower for agonists [for reviews, see Birdsall et al. (1979, 1980a), Ehlert et al. (1981), and Sokolovsky et al. (1984)]. In the absence of evidence for negative, homotropic cooperativity (Birdsall et al., 1978; Ellis & Hoss, 1980), the data are well described by a scheme in which a population of receptors is homogeneous with respect to antagonists and heterogeneous with respect to agonists. The paradox inherent in this model is clarified somewhat by reports that the binding of agonists is sensitive in a noncompetitive fashion to sulfhydryl-specific reagents,

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pertussis toxin, guanylyl nucleotides, and magnesium. While the action of these agents is not fully understood, they generally appear to alter interactions between the receptor and an inhibitory G protein; the overall pattern is consistent with the notion that a receptor can exist in two or more states of affinity induced in whole or in part by the G protein and differentiated by agonists. The differences often are transparent to antagonists; under some conditions, however, antagonists also discriminate and appear to do so with a preference opposite to that of agonists (Ehlert et al., 1980; Hulme et al., 1980b, 1981; Burgisser et al., 1982; Hosey, 1982).

At least three lines of evidence suggest that the complex, binding patterns of agonists reflect the mechanism whereby the drug elicits a response. First, deviations from rectangular hyperbolic behavior generally are greater for agonists than for antagonists. Second, parameters that reflect those deviations correlate in at least some instances with characteristics of the response: if the binding patterns of agonists at muscarinic receptors in rat cerebral cortex are assumed to arise from two classes of sites, the ratio of affinities correlates with efficacy as determined from the contractile response of smooth muscle (Birdsall et al., 1977, 1978); if the binding patterns in rabbit heart are assumed to arise from a ternary complex, the affinity of the G protein for the receptor correlates with efficacy as determined from the inhibition of adenylate cyclase (Ehlert, 1985). Third, the complexity of binding arises at least in part from the influence of a guanylyl nucleotide specific G protein that presumably links the receptor with adenylate cyclase (Smigel et al., 1983; Gilman, 1984) or other effectors (Pfaffinger et al., 1985; Breitwieser & Szabo, 1985). These considerations suggest that all states of affinity recognized by agonists lie in the mechanistic pathway that leads ultimately to a pharmacological response; accordingly, both the number of receptors in one or the other state and the cycling of receptors through the different states may be relevant to transduction of the neurohumoral signal. The identification of a model that is consistent with the binding patterns revealed by agonists thus is a useful strategy in the elucidation of neurohumoral mechanisms.

Cardiac muscarinic receptors have proven instrumental in efforts to account for the complex binding patterns of agonists since, as noted by Berrie et al. (1979), the action of guanylyl nucleotides is more pronounced in the heart than in the brain. The implication of a nucleotide-specific G protein has prompted many investigators to interpret their data in terms of a ternary complex model often cited to rationalize the binding properties of neurohumoral receptors linked to adenylate cyclase (Jacobs & Cuatrecasas, 1976; De Lean et al., 1980). In most reports, however, this model has been used only as a basis for discussion and not applied in any quantitative manner; rather, experimental data typically have been analyzed assuming that agonists bind to a heterogeneous population of noninterconverting sites. Parameters extracted in this fashion then have been taken as relevant to a system in which the receptor is in reversible association with a nucleotide-specific G protein. The report of Lee et al. (1986) demonstrates, however, that the two models are not equivalent; the present results suggest that neither model describes the binding of agonists to cardiac muscarinic receptors. Preliminary reports of this work have appeared elsewhere (Wong et al., 1981; Wells et al., 1982).

Materials and Methods

Muscarinic Drugs. *N*-[^{3}H]Methylscopolamine either was synthesized as described previously (Hulme et al., 1978) from [^{3}H]methyl iodide (10.0 Ci/mmol, New England Nuclear) and scopolamine (Sigma) or was purchased from New England Nuclear (81.8 and 83.3 Ci/mmol). All samples were assayed for purity from time to time by using thin-layer chromatography on silica gel (Whatman LK6DF) in 1-butanol/acetic acid/water (5:3:3; Hulme et al., 1978). The material was not used if the radioactivity of the major peak represented less than 93% of that found on the chromatogram.

Oxotremorine M was generously donated by Dr. N. J. M. Birdsall of the National Institute for Medical Research, London, U.K., and trihexyphenidyl by L. Dan of Novopharm Limited, Toronto. Furmethide was the gift of Smith Kline and French Canada Limited, Toronto, and was made available through the cooperation of H. A. Sheppard. All other muscarinic drugs were purchased from Sigma. For drugs obtainable only as racemic mixtures (atropine, *N*-methylatropine, trihexyphenidyl, methacholine, and bethanechol), concentrations have been corrected by assuming that one isomer is inactive.

Other Chemicals. Solvents used for thin-layer chromatography of *N*-[^{3}H]methylscopolamine were obtained as reagent grade and distilled prior to use. Guanylyl imidodiphosphate (GMP-PNP)[1] was purchased from Boehringer-Mannheim. All other materials were reagent grade or better and were used without further purification.

Membrane Preparations. Syrian golden hamsters (Bio R.B.) were obtained from a commercial breeder (TELACO, Bar Harbor, ME) and allowed to acclimatize for at least 2 weeks. The animals were killed by decapitation, and the hearts were flushed with ice-cold sucrose (0.32 M) prior to dissection into the three regions investigated: left ventricle plus interventricular septum, right ventricle, and left plus right atria. Subsequent procedures all were carried out at 2–4 °C. Tissue from 12–30 animals was pooled and homogenized in sucrose (0.32 M) using a Brinkmann Polytron (setting 8, 20 s) followed by a Potter–Elvehjem tissue blender with a Teflon pestle. The homogenate was divided into fractions containing sufficient protein for one experiment and centrifuged for 30 min at 113000*g*. Pellets were stored at −75 °C until required for use. To prepare homogenates for binding assays, a Potter–Elvehjem blender was used to resuspend the pellets in Krebs–Henseleit buffer modified by the substitution of NaHEPES (Calbiochem Ultrol, 12 mM, pH 7.48) for carbonate/bicarbonate. The suspensions then were passed through three layers of cheesecloth and diluted to achieve the concentration of protein required for the assay. Protein was determined by the method of Lowry et al. (1951) and adjusted to a concentration of 0.5–1.0 mg/mL for the binding assays. Bovine serum albumin was used as the standard.

Binding Assays. In all experiments, bound radioactivity was separated by microcentrifugation in a procedure similar to that described by Hulme et al. (1978). Following preincubation at 30 °C for 15 min, aliquots (490 μL) of the homogenate were added to polypropylene microcentrifuge tubes (500-μL capacity) containing the radiolabeled antagonist and any other drugs, each dissolved in 5 μL of water at 100-fold the required concentration. The suspension was allowed to equilibrate at 30 °C for 45 min and centrifuged at 8700*g* for 5 min (Beckman Microfuge B). The pellet was superficially washed 3 times with 0.1 M NaCl and dissolved overnight at 40 °C in a commercial solubilizer (NCS, Amersham). Radioactivity was counted in a nonaqueous cocktail (Beckman Ready-Solv NA) to which glacial acetic acid had been added (4 mL/L

[1] Abbreviations: GMP-PNP, guanylyl imidodiphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

of cocktail) in order to prevent chemiluminescence. Determinations were made routinely in quadruplicate and occasionally in sextuplicate. Binding experiments were of two types: direct and competitive. The former were carried out by varying the concentration of *N*-[^{3}H]methylscopolamine alone and in the presence of a large excess (0.01 mM) of the unlabeled analogue. The concentration of cold drug was sufficient to block fully the saturable component of binding, and specific binding was calculated from the difference between the two values. In competitive experiments, a fixed concentration of the radiolabeled antagonist (0.9–1.2 nM) was incubated with various concentrations of an unlabeled drug. Estimates of maximal and minimal binding were obtained in control assays where the radioligand was the only muscarinic drug and where specific binding was precluded by 0.01 mM unlabeled *N*-methylscopolamine.

Assay of Radioactivity. All samples were counted twice for 5 min in a Beckman liquid scintillation spectrometer (Model LS9100 or LS7500). The rate of disintegration was determined for each sample by using an external standard. Replicate samples and duplicate counts then were averaged to obtain the mean value used in subsequent analyses. Standard errors of the mean for four replicates counted twice generally were less than 1% and thus fall within the area of the symbols in subsequent figures.

Analysis of Data. All data were analyzed with total binding taken as the dependent variable (B_{obsd} in curies per milliliter). The inhibitory profiles of unlabeled drugs (A) can be described empirically by eq 1, in which n_H is the Hill coefficient and IC_{50}

$$B_{obsd} = (B_{max} - B_{min})\frac{IC_{50}^{n_H}}{[A]^{n_H} + IC_{50}^{n_H}} + B_{min} \quad (1)$$

is the concentration of A that inhibits specific binding by 50%. B_{max} and B_{min} represent total binding of the radioligand at [A] = 0 and [A] $\rightarrow \infty$, respectively. Variable parameters during the iterative, fitting procedure were B_{max}, B_{min}, n_H, and IC_{50}.

Apparent dissociation constants were extracted from the data by assuming a model in which all muscarinic drugs are fully competitive at a common population of sites. The binding patterns of antagonists were analyzed according to eq 2, in

$$B_{obsd} = \left\{\frac{[R]_t([P]_t - [RP])}{([P]_t - [RP]) + K_P\{1 + [([A]_t - [RA])/K_A]\}} + C([P]_t - [RP])\right\}SA \quad (2)$$

which $[P]_t$ and $[A]_t$ represent the total concentrations of *N*-[^{3}H]methylscopolamine and unlabeled antagonist, respectively; K_P and K_A are the equilibrium dissociation constants of P and A. $[R]_t$ is the total concentration of receptors, [RP] and [RA] are the concentrations of drug–receptor complex, *C* is the fraction of free *N*-[^{3}H]methylscopolamine that ultimately appears as nonspecific binding, and SA is the specific activity of the radioligand. The expression is a cubic polynomial in RP. Total rather than free concentrations were used in order to avoid artifacts that arise with drugs of high affinity where the dissociation constant can be comparable to the concentration of receptors (Jacobs et al., 1975; Chang et al., 1975). For direct experiments, K_A was fixed arbitrarily at a value consistent with greater than 99.99% inhibition of the radioligand in the presence of 0.01 mM unlabeled *N*-methylscopolamine, and estimates of K_P, $[R]_t$, and *C* were obtained by regression. For competitive experiments, K_P was set at 0.385 nM (log K_P = −9.414) as described below, and estimates of K_A, $[R]_t$, and *C* were obtained by regression. Control studies by equilibrium dialysis confirmed that nonspecific binding arises solely from physical entrapment of the radioligand within the pellet.

The binding patterns of agonists were analyzed according to eq 3 (Wells et al., 1980), in which the total binding is the sum of *n* saturable components plus the nonspecific component.

$$B_{obsd} = \left\{[R]_t \sum_{S=1}^{n} \left\{\frac{F_S}{1 + K_P/([P]_t - [RP]) + K_P[A]_f/[K_S([P]_t - [RP])]}\right\} + C([P]_t - [RP])\right\}SA \quad (3)$$

F_S is the fraction of total receptors characterized by the equilibrium dissociation constant K_S for the interaction between the unlabeled ligand and sites of type *S*. The radiolabeled probe (P) is assumed to exhibit the same affinity (K_P) at all sites. Since K_S exceeds $F_S[R]_t$ by at least 2 orders of magnitude for all agonists, free rather than total concentrations of the unlabeled drug could be used ($[A]_f$), and the expression is a polynomial of order $n + 1$ with respect to RP. The value of *n* was set arbitrarily, and estimates of K_S, F_S, $[R]_t$, and *C* were obtained by regression. Newton's method was used to obtain numerical solutions to eq 2 and 3 (Acton, 1970).

Statistical Procedures. All functions were fitted to experimental data by using an iterative procedure based on the nonlinear, least-squares algorithm of Marquardt (1963). The data were weighted according to the nature of the experiment. For studies on the inhibition of *N*-[^{3}H]methylscopolamine by unlabeled ligands, standard errors were an essentially constant percentage of the mean, and the data were weighted accordingly. This approximation is not valid, however, at the lowest concentrations of *N*-[^{3}H]methylscopolamine in experiments where the concentration of the radioligand is varied. Since all samples were counted to a constant time, and not to a constant error, the counting error is relatively high and predominates under such conditions. The measured error on replicate determinations thus tends to be a higher percentage of the mean value than at higher concentrations of the radioligand. Data in experiments involving concentrations of *N*-[^{3}H]methylscopolamine below 0.2 nM therefore were weighted according to the measured error on each point.

Statistical variation is indicated throughout by the standard error. Parametric values derived from two or more experiments generally are the means of individual values obtained from an independent analysis of each set of data; the standard errors thus reflect variability from experiment to experiment. Where only a single experiment or a single analysis of more than one experiment is involved, the standard error reflects the range of parametric values within which the sum of squares is essentially the same.

In dealing with multicomponent models such as eq 3, an arbitrary decision is required regarding the number of binding components. The addition of parameters generally will improve the fit as indicated by the sum of the squares of the residuals; a point arises, however, beyond which further additions to the model are no longer justified by the data. Our solution to this problem in part has been to characterize the distribution of data about the fitted curve by correlating neighboring residuals [Reich et al., 1972; see also Hulme et al. (1978) and Birdsall et al. (1980b)]. All agonists yield a highly significant correlation when the data are fitted by eq 3 with *n* set at 1. Binding components then can be added one

by one to the model until the correlation is no longer significant. Each addition also can be tested for the significance of its effect on the variance of residuals.

All parametric values and the fitted curves in all figures were obtained by fitting the appropriate function to measured estimates of total binding expressed in units of radioactivity per milliliter of reaction mixture; for eq 2 and 3, a scaling factor (SA) reflecting the specific activity of the radioligand was included in the function, and estimates of total binding were converted to molar units. Unless indicated otherwise, simultaneous analyses involving the results from competitive experiments were performed on data that previously had been normalized to 100 in the absence of unlabeled ligand and to 15 in the presence of saturating concentrations of unlabeled ligand. This procedure was dictated by variations in total binding over the course of the investigation and the attendant tendency of some experiments to dominate the variance of residuals or the correlation of neighboring residuals when data were used as collected. The mean value obtained for nonspecific binding ([A] $\rightarrow \infty$) as a fraction of specific plus nonspecific binding ([A] = 0) is 0.147 ± 0.004 for 38 experiments in left ventricular preparations; the normalization therefore has little or no effect on relative binding within individual experiments. When eq 2 or 3 was fitted to normalized data, the value of $[R]_t$ for each experiment was fixed at that obtained from an independent analysis of the data taken as measured; values of K_S, F_S, SA, and C were obtained by regression. Simultaneous analyses of data from two or more experiments generally were carried out with separate values of SA and C for each experiment; the assignment of other parameters is described where appropriate. Neighboring residuals from multiple experiments were correlated together, except that the last point from one set of data was not correlated with the first point from the next set; the variance of residuals from multiple experiments was calculated from the weighted sum of squares for all points.

The fit of individual expressions has been assessed by testing the significance of the t statistic for the correlation coefficient of neighboring residuals (Reich et al., 1972). The fits of different models, or of the same model with different sets of variable parameters, were compared by testing the significance of the F statistic for the difference in the variance of residuals (Snedecor & Cochran, 1967). All such tests were performed on weighted residuals. Levels of significance were calculated according to reported algorithms for the t statistic (Adams, 1969; Hill, 1970) and for the F statistic (Hill & Joyce, 1967; Bruning & Kintz, 1977).

Results

Binding of Antagonists. Total binding of *N*-[^{3}H]methylscopolamine reveals the same pattern in homogenates from each of the three cardiac regions studied: a nonspecific component increases linearly with the concentration of the radioligand, and a specific component can be described by assuming a single and uniform population of sites (eq 2). Three experiments were performed in order to characterize the nature of specific binding and to investigate the effect of GMP-PNP. Within each experiment, the total binding of *N*-[^{3}H]-methylscopolamine was measured in aliquots of the same homogenate under three conditions: the radioligand alone, the radioligand plus 0.01 mM unlabeled *N*-methylscopolamine, and the radioligand plus 0.1 mM GMP-PNP. Preliminary studies indicated that GMP-PNP is without effect on nonspecific binding, which therefore was measured only in its absence. Pellets used to prepare the homogenate for each experiment were from the same group of animals and were diluted to yield the same concentration of protein. Levels of binding therefore were almost identical from experiment to experiment; the residuals similarly were of comparable magnitude and could be pooled when assessing the distribution of the data about the fitted curves.

For each experiment, eq 2 can be fitted simultaneously to the data acquired in the absence and presence of unlabeled *N*-methylscopolamine, selecting for the former the measurements made either with or without GMP-PNP. The combined correlation of neighboring residuals for the three experiments is not significant either with or without the nucleotide ($P > 0.1$), and the fitted curves are in excellent agreement with the data (see supplementary material Figure 1; see paragraph at end of paper regarding supplementary material). Specific binding of the radioligand therefore can be described by assuming a single and uniform population of sites both in the absence and in the presence of GMP-PNP; the mean values of log K_P (eq 2) obtained from the three experiments are −9.39 ± 0.01 and −9.42 ± 0.01, respectively. The small decrease in K_P that accompanies the addition of GMP-PNP is negligible: neither the correlation of neighboring residuals nor the increase in the variance of residuals is significant when the results from all three experiments are analyzed simultaneously with a single value of K_P rather than with separate values for data acquired in the absence and presence of the nucleotide.

The affinity of *N*-[^{3}H]methylscopolamine shows little or no variability among the three regions studied. The mean value of log K_P is −9.33 ± 0.07 from 5 experiments in right ventricular homogenates, −9.43 ± 0.04 from 9 experiments in atrial homogenates, and −9.41 ± 0.03 from a total of 19 experiments performed in left ventricular homogenates over the course of the investigation. No value differs significantly from either of the other two. The mean value from all 33 experiments is −9.414 ± 0.020 (K_P = 385 ± 18 pM), and this has been used when required in all subsequent calculations. The specific binding of 1 nM *N*-[^{3}H]methylscopolamine in left ventricular homogenates is inhibited by the unlabeled analogue with a Hill coefficient of 0.99 ± 0.01, in good agreement with the behavior of the radioligand illustrated in supplementary material Figure 1. Moreover, analysis of the data in terms of eq 2 yields a value for log K_A of −9.41 ± 0.02 when log K_P is fixed at −9.414. The affinity of *N*-methylscopolamine determined by isotopic dilution thus is virtually identical with that determined by varying the concentration of the radioligand. Also, the binding pattern revealed by isotopic dilution is unaffected by the inclusion of 0.1 mM GMP-PNP. Estimates of K_A and $[R]_t$ derived from eq 2 (log K_P = −9.414) are virtually indistinguishable in the absence and presence of the nucleotide when both sets of data are acquired in the same experiment: the variance of residuals is not increased significantly ($P > 0.085$) nor is there a significant correlation of neighboring residuals ($P > 0.4$) when the two sets of data are analyzed simultaneously with common values of K_A, $[R]_t$, C, or all three parameters.

Maximal specific binding of the radioligand ($[R]_t$, eq 2) with animals between 150 and 250 days of age was 54–73 pmol/g of protein in the left ventricle, 81–174 pmol/g of protein in the right ventricle, and 61–96 pmol/g of protein in the combined atria. With most batches of mature animals, the density of sites found in the right ventricle was about twice that found in either the left ventricle or the combined atria. Binding also was examined in one batch of 29 animals sacrificed at 30 days of age. The density was 44 pmol/g of protein in both the combined atria and the combined ventricles, or about 40% lower than that found in most batches of older animals. No

Table I: Mean Parametric Values for the Inhibition of *N*-[^{3}H]Methylscopolamine by Agonists[a]

region and agonist	N	eq 1	eq 3						
		n_H	n	$-\log K_1$	$-\log K_2$	$-\log K_3$	F_1	F_2	F_3
left ventricle									
carbachol	16	0.65 ± 0.02	2	6.70 ± 0.06	5.25 ± 0.05		0.51	0.49 ± 0.03	
	5[c]	0.68 ± 0.02	2	6.67 ± 0.07	5.33 ± 0.08		0.46	0.54 ± 0.03	
	3[c]	0.71 ± 0.02	2	6.61 ± 0.09	5.34 ± 0.11		0.46	0.54 ± 0.04	
carbachol + GMP-PNP[b]	7	0.73 ± 0.02	2	6.83 ± 0.13	5.28 ± 0.05		0.29	0.71 ± 0.04	
	5	0.75 ± 0.02	2	6.88 ± 0.15	5.34 ± 0.04		0.26	0.74 ± 0.04	
arecoline	3	0.71 ± 0.02	2	7.23 ± 0.24	5.50 ± 0.07		0.27	0.73 ± 0.03	
oxotremorine	2[d]	0.65 ± 0.02	2	7.35 ± 0.03	5.90 ± 0.13		0.61	0.39 ± 0.02	
	5[e]	0.72 ± 0.01	3	9.19 ± 0.27	7.04 ± 0.11	6.00 ± 0.20	0.14	0.52 ± 0.08	0.34 ± 0.09
methacholine	4	0.73 ± 0.02	3	8.75 ± 0.19	6.14 ± 0.09	5.06 ± 0.04	0.09	0.68 ± 0.02	0.23 ± 0.02
oxotremorine M	2	0.67 ± 0.01	2	7.68 ± 0.23	6.01 ± 0.01		0.34	0.66 ± 0.09	
bethanechol	3	0.72 ± 0.02	2	5.88 ± 0.10	4.57 ± 0.07		0.38	0.62 ± 0.04	
furmethide	4	0.80 ± 0.01	2	5.87 ± 0.14	4.80 ± 0.07		0.38	0.62 ± 0.06	
right ventricle									
carbachol	7	0.65 ± 0.01	2	6.74 ± 0.07	5.28 ± 0.04		0.50	0.50 ± 0.03	
	5[c]	0.66 ± 0.02	2	6.72 ± 0.07	5.26 ± 0.04		0.49	0.51 ± 0.04	
atria									
carbachol	5	0.56 ± 0.02	3	8.81 ± 0.26	6.80 ± 0.10	5.27 ± 0.06	0.16	0.55 ± 0.02	0.29 ± 0.02
	3[c]	0.58 ± 0.00	3	9.18 ± 0.19	6.75 ± 0.15	5.30 ± 0.11	0.13	0.57 ± 0.03	0.30 ± 0.03
carbachol + GMP-PNP[b]	2	0.66 ± 0.03	2	6.93 ± 0.11	5.49 ± 0.02		0.46	0.54 ± 0.04	
arecoline	2	0.59 ± 0.01	2	7.38 ± 0.05	5.64 ± 0.00		0.51	0.49 ± 0.10	
oxotremorine	3	0.59 ± 0.04	2	8.04 ± 0.01	6.40 ± 0.12		0.52	0.48 ± 0.03	
methacholine	2	0.53 ± 0.00	3	7.45 ± 0.04	5.92 ± 0.03	4.39 ± 0.07	0.44	0.44 ± 0.03	0.12 ± 0.01

[a] Values listed in the table reflect best fits of eq 1 or eq 3 ($n = 2$) to the data from N experiments analyzed independently. [b] The concentration of GMP-PNP was either 0.1 or 0.6 mM, as described in the text and in footnote *b* to Table II. [c] Representative experiments selected for further analysis. For carbachol in the left ventricle, the subset of three experiments is taken from the subset of five. [d] Those experiments for which two classes of sites are adequate to describe the data. [e] Those experiments for which three classes of sites are required to describe the data.

age-dependent differences were observed in the value of K_P. Neither the capacity, expressed as picomoles per gram of protein, nor the affinity for *N*-[^{3}H]methylscopolamine was changed when left ventricular tissue from mature animals was washed extensively with ice-cold Krebs–Henseleit buffer.

The inhibition of *N*-[^{3}H]methylscopolamine by *N*-methylatropine, atropine, scopolamine, or trihexyphenidyl is consistent with the notion of a single and uniform population of sites. Hill coefficients are indistinguishable from 1 when eq 1 is fitted to data from three experiments with each antagonist: the global variance of residuals is not increased significantly ($P > 0.1$) when n_H is fixed at 1 during simultaneous analyses with no common parameters. Also, the global correlation of neighboring residuals either is negative or is not significant ($P > 0.5$) for best fits of eq 2. Mean values of n_H (eq 1) and log K_A (eq 2, log $K_P = -9.414$), respectively, are as follows: *N*-methylatropine, 0.99 ± 0.04, −8.93 ± 0.07; atropine, 1.00 ± 0.02, −8.88 ± 0.07; scopolamine, 0.93 ± 0.03, −8.64 ± 0.04; and trihexyphenidyl, 0.94 ± 0.07, −8.08 ± 0.04. In all experiments, total binding measured at saturating concentrations of the unlabeled antagonist was within 3% of that measured in the presence of 0.01 mM *N*-methylscopolamine. All antagonists thus appear to preclude access of the radioligand to the same population of sites.

Binding of Agonists. In all regions studied, saturating concentrations of any agonist tested reduced total binding of the radioligand to within 3% of that found in the presence of 0.01 mM unlabeled *N*-methylscopolamine. Agonists and antagonists thus appear to compete for a common population of sites. The inhibitory behavior of agonists can be described to a first approximation by the Hill equation (eq 1), and Hill coefficients are markedly less than 1 (Table I). Simultaneous analyses in terms of eq 3 ($n = 1$) indicate that, in every case, a single class of sites is inadequate to describe the data: the global correlation of neighboring residuals is highly significant ($P < 0.00001$), as illustrated with carbachol in Figure 1; moreover, the global variance of residuals and the variances from individual experiments are reduced substantially ($P < 0.00001$) upon the addition of a second class of sites. The levels of significance listed in Table II are based on an analysis of neighboring residuals for best fits of eq 3 with two classes of sites. Several agonists yield a poor correlation ($P > 0.1$) when the data from all experiments are taken together with no common parameters, as in the example of carbachol in the left ventricle (Figure 1A); the correlation generally is poor or only marginally significant when the values either of F_2 or of K_1 and K_2 are common to the data from different experiments. The residuals thus are distributed randomly about the fitted curve, and two classes of sites provide an adequate description of the data. Parametric values obtained from eq 3 ($n = 2$) agree well from experiment to experiment, and the mean values are listed in Table I.

A more complex pattern is observed with carbachol and methacholine in the atria and with methacholine and oxotremorine in the left ventricle. Two classes of sites yield a global correlation of neighboring residuals that is significant with no common parameters, as illustrated in Figure 1B, and highly significant with common values of F_2 or of K_1 and K_2 (Table II). The correlations observed with common parameters reflect in part a wide variability among the parametric values obtained from different experiments. With methacholine in both regions and with carbachol in the atria, three classes of sites yield excellent agreement with the data, and parametric values are consistent from experiment to experiment; the global correlation of neighboring residuals either is negative or is not significant ($P > 0.2$) irrespective of whether the data from replicate experiments are fitted with separate or common values of F_S or K_S. The mean, parametric values for three classes of sites are listed in Table I. With oxotremorine in the left ventricle, however, the addition of a third class of sites reduces the correlation coefficient of neighboring residuals but fails to yield parametric values that are consistent among the seven experiments. This discrepancy appears to reflect the absence of the sites of highest affinity from some preparations of tissue. Five experiments with homogenates from three batches of animals revealed three

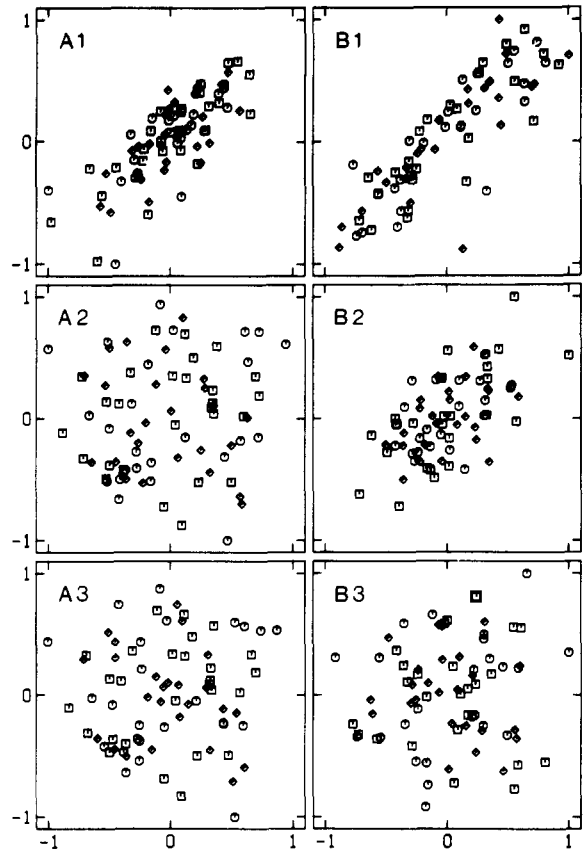

FIGURE 1: Correlation of neighboring residuals for best fits of eq 3 to the inhibitory patterns of carbachol in left ventricle (A) and atria (B). Total binding of *N*-[^{3}H]methylscopolamine (0.96–1.18 nM) was measured at different concentrations of carbachol as illustrated in Figure 4A. The data were normalized as described under Materials and Methods and analyzed in terms of eq 3 with the number of sites taken as one (A1, B1), two (A2, B2), or three (A3, B3). All parameters were assigned separate values for each experiment. Weighted residuals from three experiments were correlated with their nearest neighbors, and the correlations are illustrated in the figure. In each frame, residuals have been normalized to the largest taken as 1 or to the smallest taken as −1; different symbols identify the residuals from different experiments. Statistical parameters for correlations in the left ventricle (A) are as follows: one class of sites (A1), r = 0.77, t = 11, and $P < 0.00001$; two classes (A2), r = 0.069, t = 0.60, and P = 0.55; and three classes (A3), r = 0.045, t = 0.39, and P = 0.70. Statistical parameters for the atria (B) are as follows: one class of sites (B1), r = 0.84, t = 13, and $P < 0.00001$; two classes (B2), r = 0.57, t = 5.9, and $P < 0.00001$; and three classes (B3), r = 0.078, t = 0.66, and P = 0.51.

classes of sites, whereas two experiments with homogenates from two different batches of animals revealed only two classes (Table II); moreover, the mean values of K_2 and K_3 for the former (n = 3) compare favorably with the values of K_1 and K_2 for the latter (n = 2) (Table I). When oxotremorine and methacholine were tested in homogenates from the same batch of animals, both revealed three classes of sites; methacholine was not tested in those batches that revealed only two classes with respect to oxotremorine. Carbachol was examined at least once with every batch of tissue, however, and consistently revealed only two classes of sites in left ventricular homogenates.

The decision to include a third class of sites is supported in each instance by a substantial reduction in the variance of residuals. The decrease is significant ($P < 0.05$) for each experiment with methacholine in either region, for each experiment with carbachol in the atria, and for each of the five experiments with oxotremorine in the left ventricle; the decrease is highly significant ($P < 0.00001$) when the global variance is compared for replicates with the same agonist in the same region.

Table II: Levels of Significance (P) for the Correlation of Neighboring Residuals Obtained by Assuming Two Classes of Sites for Agonists[a]

region and agonist	N	P: no common parameters	P: common F_2	P: common K_1 and K_2
left ventricle				
carbachol	5[c]	0.13	0.086	0.0060
	3[c]	0.55[f]	0.46	0.11
carbachol + GMP-PNP[b]	7	0.29	0.046	0.00010
	5[d]	0.18	0.028	0.016
arecoline	3	0.34	0.11	0.028
oxotremorine	7	0.022	<0.00001	<0.00001
	5[e]	0.00087	0.00028	0.00005
	2[e]	0.98[g]	0.99[g]	0.68
methacholine	4	0.013	<0.00001	<0.00001
oxotremorine M	2	0.20	0.064	0.040
bethanechol	3	0.48[g]	0.52[g]	0.70[g]
furmethide	4	0.099[g]	0.16[g]	0.19[g]
right ventricle				
carbachol	7	0.17	0.044	0.0061
	5[d]	0.93[g]	0.50	0.18
atria				
carbachol	5	<0.00001	<0.00001	<0.00001
	3[d]	<0.00001[f]	<0.00001	<0.00001
carbachol + GMP-PNP[b]	2	0.46	0.22	0.43
arecoline	2	0.24	0.0035	0.19
oxotremorine	3	0.56[g]	0.55[g]	0.97[g]
methacholine	2	0.0011	0.0010	0.0012

[a] Equation 3 (n = 2) was fitted simultaneously to data from replicate experiments with each agonist. For parameters listed in the table, a single value was common to all experiments; other parameters were assigned separate values for each experiment. The correlation coefficient of neighboring residuals for the data from N experiments was computed and tested for significance as described in the text. [b] The concentration of GMP-PNP was 0.6 mM in two experiments performed in left ventricular homogenates and 0.1 mM in all other experiments; as described in the text, the inhibitory behavior of carbachol is insensitive to changes in the concentration of the nucleotide between 0.1 and 0.6 mM. [c] Sixteen experiments were performed with carbachol in left ventricular homogenates; 5 of the 16 and, in some cases, 3 of those 5 were selected as representative and used for further analysis. [d] Representative experiments selected for further analysis. [e] Among the seven experiments with oxotremorine, five reveal three classes of sites and two reveal two classes; further details are described in the text. [f] The correlation of neighboring residuals is illustrated in Figure 1. [g] The correlation coefficient of neighboring residuals is negative.

To test the validity of the notion that agonists and antagonists compete for a common population of sites, the inhibitory behavior of carbachol was compared at two concentrations of *N*-[^{3}H]methylscopolamine (Figure 2). A 5.2-fold increase in the concentration of the radioligand is accompanied by an increase of about 3-fold in the concentration of carbachol required to achieve the same level of percentage inhibition. The shift is consistent with competitive effects, since the two sets of data yield similar values of K_1, K_2, and F_2 when analyzed independently by eq 3 with log K_P fixed at −9.414; moreover, the variance of residuals is not increased significantly (P = 0.13) when the data are analyzed simultaneously with single values of K_1, K_2, and F_2 common to both curves. The inset in Figure 2 illustrates the effect of K_P on the variance of residuals obtained in simultaneous fits with common values of K_P, K_1, K_2, and F_2. At the 95% level of confidence, the variance is not significantly different with log K_P fixed at values between −9.70 and −9.35. The affinity of *N*-methylscopolamine reflected in the binding of carbachol thus is in good agreement with that measured by varying the concentration of the radioligand, as expected for competitive inhibition. Also, the failure of a 5.2-fold change in the concentration

Table III: Comparison of F_2 among Agonists Revealing Two Classes of Sites in Left Ventricular Homogenates[a]

	furmethide (4)	bethanechol (3)	oxotremorine M (2)	oxotremorine (2)[b]	arecoline (3)
carbachol (3)	0.19	0.27	0.0055	0.040	<0.00001
arecoline (3)	0.25	0.021	0.19	<0.00001[c]	
oxotremorine (2)[b]	0.024	0.014	0.00099		
oxotremorine M (2)	0.76	0.32			
bethanechol	0.66				

[a] Values listed in the table indicate the level of significance (P) for the difference in the variance of residuals (eq 3, n = 2) when the data from replicate experiments with two agonists are fitted assuming two values of F_2, one for each agonist, and a single value of F_2 for both agonists. Other parameters were assigned separate values for each experiment. The number of experiments is indicated in parentheses. [b] The comparison includes only those experiments in which oxotremorine revealed two classes of sites. [c] The corresponding value of P in atrial homogenates is 0.56 for a comparison involving two replicates with arecoline and three replicates with oxotremorine.

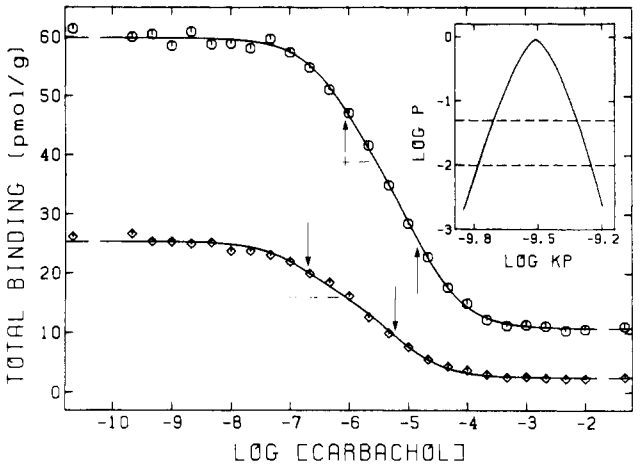

FIGURE 2: Inhibition of N-[^{3}H]methylscopolamine by carbachol at two concentrations of the radioligand. Total binding of N-[^{3}H]-methylscopolamine (○, 1.18 nM; ◇, 0.23 nM) was measured in homogenates of left ventricle (○, 0.59 mg of protein/mL; ◇, 0.63 mg of protein/mL) at the concentrations of carbachol shown on the abscissa. Tissue from the same batch of animals was used in both experiments. The lines represent best fits of eq 3 (n = 2) to the experimental data; the value of log K_P was set at −9.414 as described in the text. Parametric values obtained by regression are as follows: (○) log K_1 = −6.67 ± 0.11, log K_2 = −5.46 ± 0.07, F_2 = 0.57 ± 0.06, $[R]_t$ = 65.8 ± 0.4 pmol/g of protein, and C = 0.00529 ± 0.00007; (◇) log K_1 = −6.91 ± 0.15, log K_2 = −5.44 ± 0.08, F_2 = 0.58 ± 0.06, $[R]_t$ = 64.8 ± 0.8 pmol/g of protein, and C = 0.0067 ± 0.0002. Points at the lower and upper limits of the abscissa indicate binding in the absence of agonist and in the presence of 0.01 mM unlabeled N-methylscopolamine, respectively. The arrows indicate the concentration of carbachol that inhibits specific binding by 50% at each class of sites, and the dashed lines indicate the relative size of each class. The results presented in the inset derive from a series of simultaneous analyses (eq 3, n = 2) in which the two sets of data shared common values of K_P, K_1, K_2, and F_2; each set was assigned separate values of SA and C. Values plotted on the ordinate indicate the level of significance (P) for the difference in the variance of residuals between that obtained with the optimal value of K_P, as determined by regression, and that obtained with K_P fixed at the value shown on the abscissa. Other parameters were determined by regression. The dashed lines indicate 95% and 99% levels of confidence.

of N-[^{3}H]methylscopolamine to alter the value of F_2 concurs with the observation that all sites bind the radioligand with the same affinity.

The model described by eq 3 implies that the sites exhibiting one or the other affinity for an agonist cannot interconvert. All agonists therefore are expected to reveal the same number of classes (n) and the same, relative number of sites (F_S) within each class. The first prediction is at variance with the observation that n is 2 for some agonists and 3 for others in both the atria and the left ventricle. The difference might be rationalized if some agonists were to exhibit the same or similar affinity for the sites of two classes and a different affinity for those of the third. All agonists nevertheless are expected to yield the same value of F_2 when n is 2 or the same values of F_2 and F_3 when n is 3.

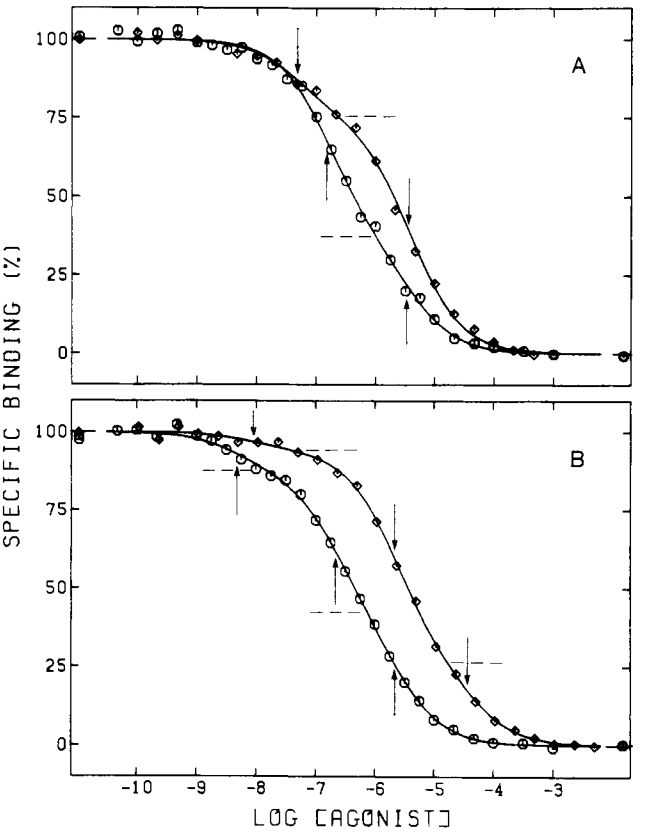

FIGURE 3: Inhibition of N-[^{3}H]methylscopolamine by agonists that differ in the relative capacities revealed for each class of sites. Total binding of N-[^{3}H]methylscopolamine (1.04–1.22 nM) was measured in homogenates of left ventricle at the concentrations of agonist shown on the abscissa. Data plotted in panels A and B are for agonists that reveal two and three classes of sites, respectively. The lines represent best fits of eq 3 (n = 2 or 3) to the experimental data. Parametric values obtained by regression in panel A are as follows: for oxotremorine (○), log K_1 = −7.38 ± 0.07, log K_2 = −6.03 ± 0.10, and F_2 = 0.37 ± 0.05; for oxotremorine M (◇), log K_1 = −7.91 ± 0.17, log K_2 = −6.00 ± 0.04, and F_2 = 0.76 ± 0.03. Parametric values in panel B are as follows: for oxotremorine (○), log K_1 = −8.85 ± 0.26, log K_2 = −7.20 ± 0.18, log K_3 = −6.22 ± 0.12, F_2 = 0.46 ± 0.08, and F_3 = 0.41 ± 0.10; for methacholine (◇), log K_1 = −8.60 ± 0.38, log K_2 = −6.23 ± 0.06, log K_3 = −5.01 ± 0.11, F_2 = 0.68 ± 0.04, and F_3 = 0.26 ± 0.04. Values plotted on the ordinate are normalized at 100% and 0% to the asymptotic values of eq 3 obtained from the fitting procedure. Further details are given in the legend to Figure 2.

Values of F_2 listed in Table I vary from 0.39 to 0.73 for the six agonists that reveal two classes of sites in the left ventricle. Data for oxotremorine and oxotremorine M are compared in Figure 3A. Differences in F_2 between pairs of agonists have been tested for significance by simultaneous analysis in which the data are fitted either with two values, one for all replicates with each agonist, or with a single value common to all replicates with both agonists. The comparison assumes that all agonists exhibit the same preference for the two classes of sites. Among the 15 possible combinations (Table III), the increase

in the variance of residuals that occurs with a single value of F_2 is significant in 8 ($0.05 > P > 0.005$) and highly significant in 3 ($P < 0.001$). The differences suggest that the number of receptors exhibiting one or the other affinity is controlled at least in part by the agonist. Similar behavior is observed with agonists that reveal three classes of sites (see supplementary material Table I). When methacholine is compared with oxotremorine in the left ventricle (Figure 3B) or with carbachol in the atria, the variance of residuals is found to increase significantly ($P < 0.00001$) when the two agonists share common values of both F_2 and F_3. In the left ventricle, the variance is essentially unchanged when F_1 is the only parameter common to both agonists ($P = 0.60$), but it is increased with a common value of either F_2 ($P = 0.036$) or F_3 ($P = 0.049$). In the atria, the variance is increased significantly with a common value of F_1, F_2, or F_3 ($P < 0.004$). The differences in F_S between agonists that reveal three classes of sites argue against the possibility that differences between agonists that reveal only two classes (Table III) arise from inadequately resolved binding patterns.

Carbachol exhibits the same Hill coefficient and inhibitory potency (IC_{50}, eq 1) in both the left and the right ventricle. The similarity is reflected in estimates of affinity and relative capacity derived from eq 3 ($n = 2$) (Table I); also, the data from both regions can be fitted with common values of the various parameters with little or no change in the variance of residuals ($P > 0.05$, Table IV). In contrast, Hill coefficients for carbachol and three other agonists are substantially lower in the atria than in the left ventricle (Table I); estimates of IC_{50} (eq 1) are 2.9–4.5-fold lower in the atria. Arecoline reveals two classes of sites in both regions when the data are analyzed in terms of eq 3 (Table II). The parametric values (Table I) and the effects of common parameters on the variance (Table IV) suggest that the difference reflects a larger fraction of atrial sites in the class of higher affinity; there apparently is no difference in the affinity of the agonist for the sites of either class (Figure 4B). A similar pattern emerges with carbachol (Tables I and IV). The sites of highest affinity (K_1) are not observed in the left ventricle, and there is no regional difference in the values of K_2 and K_3 for the sites of intermediate and lowest affinity, respectively. About 50% of the sites are of intermediate affinity in both regions, but the data are not in accord with a common capacity for the sites of lowest affinity ($P < 0.00001$, Table IV).

The comparisons with arecoline and carbachol suggest that regional differences in binding reflect differences in the relative numbers of receptors within three classes, at least two of which are common to both regions studied. Similar consistency is not found, however, with either methacholine or oxotremorine. Methacholine reveals three classes of sites in both the atria and the left ventricle (Table I), as illustrated in Figure 4C, but there is a major increase in the variance of residuals if K_1, K_2, and K_3 all are presumed common to both regions ($P < 0.00001$; Table IV). If two of the three affinities are presumed common, the increase is small ($P = 0.028$) only in the case of K_2 and K_3. With a single common parameter, the increase is negligible only in the case of K_2 ($P = 0.13$). The analyses summarized in Table IV cover only those possibilities in which K_1 in the atria is compared with K_1 in the left ventricle, K_2 is compared with K_2, and K_3 with K_3. Other arrangements either converge on one of the minima reflected in Table IV or result in substantial increases in the variance ($P < 0.00001$). A total of at least four and possibly five classes of sites thus are required to rationalize the behavior of methacholine in terms of eq 3. A similar result emerges with oxotremorine, where the two affinities observed in the atria cannot be linked with any two of the three affinities observed in the left ventricle without a substantial increase in the variance of residuals ($P < 0.004$). At least four classes of sites thus are required to rationalize the behavior of oxotremorine.

Table IV: Regional Comparison of Parametric Values for the Binding of Agonists[a]

agonist	region[b] A	region[b] B	common parameter(s)[c]	P
carbachol	LV(5), $n = 2$	RV(5), $n = 2$	K_1, K_2	0.053
			F_2	>0.99
			K_1, K_2, F_2	0.11
arecoline	AT(2), $n = 2$	LV(3), $n = 2$	K_1, K_2	0.16
			F_2	0.00002
carbachol	AT(5), $n = 3$	LV(5), $n = 2$	K_1, K_2[d]	<0.00001
			K_1, K_3[e]	
			K_2, K_3[f]	0.033
			K_2[f]	0.17
			K_3[f]	0.73
			K_2, K_3, F_2[f]	0.060
			K_2, K_3, F_3[f]	<0.00001
methacholine	AT(2), $n = 3$	LV(4), $n = 3$	K_1, K_2, K_3	<0.00001
			K_1, K_2	0.00001
			K_1, K_3	<0.00001
			K_2, K_3	0.028
			K_1	<0.00001
			K_2	0.13
			K_3	0.0098
oxotremorine	AT(3), $n = 2$	LV(5), $n = 3$	K_1, K_2[g]	0.00067
			K_1, K_3[h]	0.0012
			K_2, K_3[i]	0.0038
			K_2[i]	0.010
			K_3[i]	0.42

[a] Equation 3 ($n = 2$ or 3) was fitted simultaneously to data from replicate experiments in homogenates of two regions (A and B). Parameters listed in the table were common to all experiments in both regions or, in a separate analysis, to only those experiments in the same region; levels of significance (P) reflect the increase in the variance of residuals with one rather than two values of the parameters shown. Parameters not listed in the table either were common to experiments in the same region (K_S and F_S) or were assigned separate values for each experiment (SA and C). [b] Abbreviations: LV, left ventricle and interventricular septum; RV, right ventricle; AT, left plus right atria. The number of individual experiments is shown in parentheses. The number of classes of sites (n) assigned to each region (A or B) was determined as described in the text and is based on the data summarized in Table II. The total number of sites assigned to both regions (A + B) depends in part upon the values of K_S that are held in common; with methacholine, for example, the atria and left ventricle together contain five classes of sites if only one class is common to both regions. [c] Parameters within each region are identified by subscripts in order of decreasing affinity of the sites for the agonist (i.e., $K_1 < K_2 < K_3$). Carbachol and oxotremorine both reveal more classes of sites in one region than in the other, and parameters are numbered according to the region with the greater multiplicity. [d] F_3 was fixed at zero for data from the left ventricle. [e] F_2 was fixed at zero for data from the left ventricle; convergence was to parametric values identical with those obtained with F_1 fixed at zero. [f] F_1 was fixed at zero for data from the left ventricle. [g] F_3 was fixed at zero for data from the atria. [h] F_2 was fixed at zero for data from the atria. [i] F_1 was fixed at zero for data from the atria.

Effect of GMP-PNP on Binding of Carbachol. The binding of *N*-methylscopolamine is not affected by GMP-PNP in the preparations used in the present investigation. In contrast, the Hill coefficient for carbachol is increased from 0.65 to about 0.73 in the left ventricle and from 0.56 to 0.66 in the atria (Table I); the inhibitory potency (IC_{50}, eq 1) is increased about 2.3-fold in both regions. The binding patterns remain too shallow, however, to be described by eq 3 with only a single class of sites: the correlation of neighboring residuals is highly significant both in individual experiments ($P < 0.00004$) and

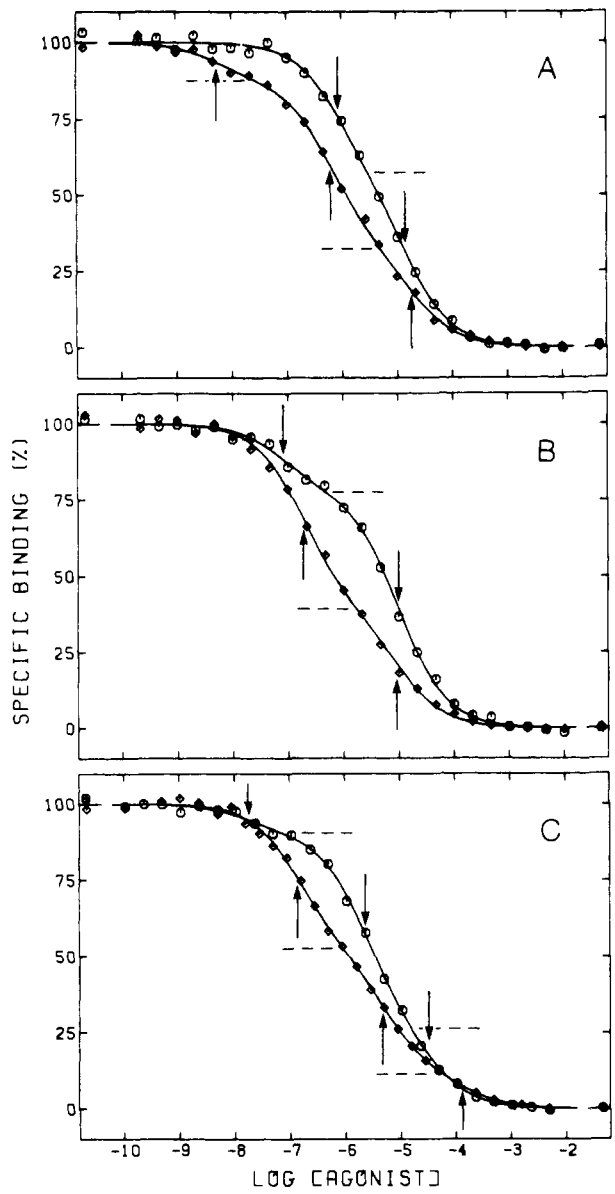

FIGURE 4: Regional differences in the inhibition of *N*-[^{3}H]methylscopolamine by agonists. Total binding of *N*-[^{3}H]methylscopolamine (0.96–1.28 nM) was measured in homogenates of left ventricle (○) and atria (◇) at the concentrations of carbachol (A), arecoline (B), or methacholine (C) shown on the abscissa. The lines represent best fits of eq 3 to the experimental data. Parametric values obtained by regression are as follows: (A) carbachol in the left ventricle (○, n = 2), log K_1 = −6.67 ± 0.11, log K_2 = −5.46 ± 0.07, and F_2 = 0.57 ± 0.06; carbachol in the atria (◇, n = 3), log K_1 = −8.81 ± 0.34, log K_2 = −6.78 ± 0.11, log K_3 = −5.35 ± 0.09, F_2 = 0.52 ± 0.04, and F_3 = 0.36 ± 0.04; (B) arecoline in the left ventricle (○, n = 2), log K_1 = −7.71 ± 0.15, log K_2 = −5.63 ± 0.03, and F_2 = 0.78 ± 0.02; arecoline in the atria (◇, n = 2), log K_1 = −7.33 ± 0.06, log K_2 = −5.64 ± 0.06, and F_2 = 0.38 ± 0.02; (C) methacholine in the left ventricle (○, n = 3), log K_1 = −8.38 ± 0.24, log K_2 = −6.26 ± 0.08, log K_3 = −5.12 ± 0.12, F_2 = 0.65 ± 0.05, and F_3 = 0.26 ± 0.05; methacholine in the atria (◇, n = 3), log K_1 = −7.41 ± 0.05, log K_2 = −5.89 ± 0.09, log K_3 = −4.45 ± 0.15, F_2 = 0.41 ± 0.02, and F_3 = 0.11 ± 0.02. Further details are given in the legend to Figure 3.

when the data from all experiments from one or the other region are correlated together ($P < 0.00001$); also, the variance of residuals is reduced substantially ($P < 0.00001$) upon the addition of a second class (n = 2). Two classes of sites are sufficient to provide good descriptions of the data from both regions (Table II). The inhibition corresponding to F_1 is small in the left ventricle, however, and K_1 is associated with a relatively large, parametric error.

Two classes of sites are observed in the left ventricle with or without GMP-PNP, which increases the value of F_2 ($P < 0.00001$) but has little or no effect on K_1 or K_2 ($P = 0.10$) (Table I and supplementary material Table II). Sites of higher affinity for carbachol that coexist with GMP-PNP appear not to reflect a subsaturating concentration of the latter. The concentration of nucleotide was 0.6 mM in two of the seven experiments summarized in Table I and 0.1 mM in the other five. Both experiments at the higher concentration and one experiment at the lower were performed with tissue from the same batch of animals. The variance of residuals is not increased significantly when either set of data at 0.6 mM GMP-PNP and that at 0.1 mM GMP-PNP are fitted simultaneously with single rather than separate values of F_2 ($P > 0.07$), K_1 and K_2 ($P > 0.17$), or K_1, K_2, and F_2 ($P > 0.26$). In no instance is there a significant correlation of neighboring residuals ($P > 0.05$). A 6-fold increase in the concentration of the nucleotide thus is without effect on the inhibitory behavior of carbachol. It follows that the sites of higher affinity that coexist with GMP-PNP reflect a limiting or asymptotic state of the system and not merely an insufficient concentration of the nucleotide.

The inclusion of GMP-PNP reduces the number of classes of sites observed in the atria from three to two (Table II). As in the left ventricle, this change appears to reflect a redistribution of sites from one class to another. The apparent dissociation constants at the sites of intermediate and lowest affinity (K_2 and K_3) observed in the absence of GMP-PNP agree closely with those at the sites of higher and lower affinity (K_1 and K_2) observed in the presence of GMP-PNP (Table I). The similarity is confirmed by the negligible increase in the variance of residuals ($P = 0.28$) when the sites apparently common to both conditions share common values of K_S (supplementary material Table II); other combinations of K_S either result in a markedly higher variance of residuals ($P < 0.00001$) or converge to yield the arrangement of parameters described above. The sites ostensibly of intermediate affinity (K_2) for carbachol in the atria constitute approximately 50% of the labeled sites either with or without GMP-PNP (Table I and supplementary material Table II). The nucleotide thus appears to convert the sites of highest affinity to sites of lowest affinity with little effect on the sites of intermediate affinity, although other rearrangements also are consistent with the data. There is a substantial increase in the variance ($P < 0.00001$) if the fraction of sites ostensibly of lowest affinity is taken as common to both conditions (supplementary material Table II).

Discussion

The saturable binding of *N*-methylscopolamine to preparations used in the present investigation reveals a single and uniform population of sites. Excellent descriptions of the data are provided by eq 2 irrespective of whether the radioligand or the unlabeled analogue is taken experimentally as the independent variable. Both methods yield a value of −9.41 for log K_P, which in turn is indistinguishable from the value of −9.50 that can be inferred from the effect of a 5.2-fold increase in the concentration of *N*-[^{3}H]methylscopolamine on the inhibitory behavior of carbachol. Moreover, neither the shape of the binding pattern nor the estimate of K_P is sensitive to GMP-PNP, again irrespective of whether the concentration of labeled or unlabeled *N*-methylscopolamine is varied. The complex behavior of agonists and the attendant sensitivity to guanylyl nucleotides thus arise from a phenomenon that is transparent to *N*-methylscopolamine under the present conditions. It follows that the radioligand labels a random sample

from the total population of receptors in all experiments. The inhibitory patterns of agonists therefore are likely to mirror the binding patterns that would be obtained were it possible to investigate such drugs directly. A useful consequence of this situation relates to the influence of the antagonist on the observed behavior of agonists. It is implicit in both the multisite model (eq 3) and the ternary model discussed below that agonists and antagonists compete for a single site on the receptor; the binding properties of *N*-methylscopolamine thus are sufficient in themselves to justify the relatively simple extrapolation whereby one obtains parametric values unique to the agonist. Not all models behave similarly when the radioligand binds in a rectangular hyperbolic manner. Mattera et al. (1985) have pointed out, for example, that the radioligand may be cooperative with respect to agonists but not with respect to itself in systems that can be described by the Adair equation.

There is considerable uncertainty over the phenomenon underlying the low, Hill coefficients for agonists, although much evidence points to the involvement of an inhibitory G protein. In the absence of data favoring alternative models, most investigators have adopted a scheme wherein sites of two or more different classes differ in their affinity for the drug. The data typically are analyzed according to eq 3 or equivalent expressions, with the attendant implication that agonists are without effect on the distribution of receptors among the various classes. Such a scheme is at variance with the binding patterns measured for agonists in the present investigation. Within either the left ventricle or the atria, two classes of sites are adequate to describe the inhibitory behavior of some agonists while three classes are required for others; furthermore, agonists that reveal the same degree of multiplicity within either region differ significantly in their values of F_2 ($n = 2$) or of F_2 and F_3 ($n = 3$). Of particular interest are differences in F_S between agonists that reveal three classes of sites, since one presumably is approaching the limit of resolution for binding patterns that span the range of concentrations found with neurohumoral agonists in most systems. Equation 3 therefore is compatible with the data for single agonists taken individually but incompatible with those for all agonists taken together unless the number of classes of receptor exceeds three.

A similar conclusion can be drawn when binding of the same agonist is compared in atrial and ventricular preparations. With separate classes of noninterconverting sites, regional differences might be expected to arise from differences in the relative numbers of sites in one or other classes; indeed, Birdsall et al. (1980b) have reported that such an interpretation can account for differences in the binding of carbachol to homogenates prepared from six regions of rat brain. In the present investigation, both carbachol and arecoline yield estimates of K_S that are indistinguishable in atria and left ventricle; differences in binding can be attributed exclusively to differences in F_S, in agreement with the observations of Birdsall et al. (1980b). This regional similarity does not extend to methacholine, for which differences in K_S suggest that at least one and probably two classes of receptor are unique to each region. Since three classes are observed in both regions, at least four and probably five classes are required to describe the system. A similar pattern is observed with oxotremorine. The regional differences in K_S found with methacholine and oxotremorine suggest that the similarities found with carbachol and arecoline are fortuitous. The present data therefore argue that several states of affinity are accessible to cardiac muscarinic receptors and that the distribution of receptors among the various states is determined at least in part by muscarinic agonists. The possibility of an agonist-mediated interconversion of receptors from one state of affinity to another calls into question the physical significance of all parameters derived from eq 3 and particularly that of F_S. A binding pattern is defined by progressive increases in the concentration of an agonist; values of F_S thus may not bear any simple relationship to the number of receptors in one or other states of affinity at any particular point in the curve.

Values of F_2 ($n = 2$) that differ among agonists have been reported previously, although the differences may not always be significant or may reflect the choice of two rather than three classes of sites [see, for example, Waelbroeck et al. (1982) and Jim et al. (1982)]. Burgisser et al. (1982) found that F_2 is 0.3 for the agonist oxotremorine and 0.5 for the antagonist quinuclidinyl benzilate in homogenates of whole frog hearts under conditions where both compounds reveal a Hill coefficient less than 1. In contrast, different agonists are reported to reveal the same value of F_S for each class of sites in homogenates of rat cerebral cortex (Birdsall et al., 1978, 1980b). This difference may be related to the observation that the effect of guanylyl nucleotides on the binding of agonists is much smaller in the cortex than in the heart of both rat (Hulme et al., 1980b) and hamster (Wells et al., 1984).

The action of guanylyl nucleotides on cardiac muscarinic receptors implies an interaction between the receptor and an inhibitory G protein. Moreover, Kent et al. (1980) have demonstrated that β-adrenergic receptors in frog erythrocyte membranes reveal a heterogeneity similar to that found in the present study; that is, different agonists reveal different numbers of receptors in states of higher or lower affinity. De Lean et al. (1980) have reported that the binding patterns of adrenergic agonists can be described quantitatively by assuming that agonist, receptor, and a third component likely to be the G protein combine to yield a ternary complex in the manner proposed, for example, by Jacobs and Cuatrecasas (1976). In view of the similarities between muscarinic and β-adrenergic receptors, the scheme of De Lean et al. (1980) has been assessed for its relevance to the present data.

Four quantities control the binding of agonists in the ternary model (Lee et al., 1986): (a) the ratio of total G protein to total receptor within the membrane ($[G]_t/[R]_t$); (b) the relative affinity of the agonist (A) for the receptor alone (R) and for the complex (RG) between receptor and G protein (K_A/K_{AG}); (c) the relative affinity of the radiolabeled antagonist (P) for the two forms of the receptor (K_P/K_{PG}); and (d) the total concentration of receptor within the membrane relative to its affinity for the G protein ($[R]_t/K_G$). It is implicit in the model that agonists are without effect on either K_G or $[G]_t/[R]_t$. Several assumptions are made in the analysis of the present data. First, all receptors in the pool labeled by *N*-[^{3}H]methylscopolamine are assumed to be accessible to all relevant G proteins; that is, none of the labeled receptors is sequestered in a compartment to which G proteins are denied access. Such an assumption seems reasonable, since compartmentalized receptors would be expected to exhibit behavior generally in agreement with the predictions of eq 3. Second, the G protein is considered to be without effect on the interaction between the radioligand and the receptor ($K_P = K_{PG}$); this follows from the observation that the specific binding of *N*-methylscopolamine is well described by eq 2 and insensitive to GMP-PNP. Third, evidence suggests that agonists bind with higher affinity to RG than to R [see, for example, Cerione et al. (1984) and Florio & Sternweis (1985)]; K_A/K_{AG} therefore is presumed to exceed 1. As pointed out by Lee et

al. (1986), however, the model also predicts that agonists could bind with higher affinity to R than to RG ($K_A/K_{AG} < 1$).

A further complication associated with fitting the ternary model arises from the relationship between the shape of the binding curve and the relative affinity of the agonist for R and for RG. As pointed out by Lee et al. (1986), empirical parameters such as n_H (eq 1) or K_2/K_1 (eq 3, $n = 2$) become independent of K_A/K_{AG} at values of the latter that lie outside a range determined by the value of $[R]_t/K_G$. K_{AG} thus may be highly correlated with K_G, which is bounded only by an upper limit on $[R]_t/K_G$ of about 0.1 in the case of the present data. The variance of residuals is increased only at values of K_G that yield higher values of $[R]_t/K_G$; it otherwise is independent of K_G for most agonists, as are the values of K_A and $[G]_t/[R]_t$ that yield an optimal fit. In order to avoid the correlation between K_{AG} and K_G associated with an optimal fit, the latter has been fixed throughout at a value that corresponds to estimates of $[R]_t/K_G$ at least 1000-fold below the acceptable maximum of about 0.1.

If agonists are indeed without effect on total G or total R, and if the ternary model is relevant to the present data, several considerations suggest that the ratio of $[G]_t$ to $[R]_t$ is near or equal to 1. The studies of Lee et al. (1986) indicate that a ratio of 1 places a lower limit of 0.67 on the Hill coefficient irrespective of K_A/K_{AG} or $[R]_t/K_G$. This limit increases to 0.85 with only a 1.4-fold excess of G proteins and to 0.9 with a 2-fold excess. Since experimental estimates of the Hill coefficient are as low as 0.65 in the left ventricle and 0.52 in the atria, an excess of G proteins is precluded in both regions. Lee et al. (1986) also have shown that systems with an excess of receptors tend to reveal two classes of sites for agonists when the data are analyzed in terms of eq 3. Predicted values of F_2 are essentially the same when $[R]_t$ exceeds K_G and show some variability when $[R]_t$ is comparable to or less than K_G. Values of F_2 listed in Table I vary from 0.39 to 0.74 among agonists that reveal two classes of sites in the left ventricle; the spread of about 0.35 places a lower limit of about 0.8 on the ratio of $[G]_t$ to $[R]_t$ [cf. Figure 7 in Lee et al. (1986)]. For a relatively small excess of receptors over G proteins, the investigation of Lee et al. (1986) suggests that some agonists might reveal three classes of sites (eq 3) if K_A differed sufficiently from K_{AG} and if the data were of sufficient quality and resolution. The sites ostensibly of lowest ($K_A/K_{AG} > 1$) or highest ($K_A/K_{AG} < 1$) affinity reflect the extra receptors and are expected to appear similar in number for all agonists at any value of $[R]_t/K_G$. This prediction is not consistent with the observation that F_3 differs between agonists that reveal three classes of sites (supplementary material Table I). The data therefore argue that G protein and receptor must be present in equimolar or nearly equimolar amounts if events at the receptor are to be described by the ternary model.

With $[G]_t/[R]_t$ fixed at 1, and with log K_P and log K_{PG} both fixed at −9.414, best fits of the ternary model fail to describe the binding patterns obtained for agonists in left ventricular homogenates.[2] When replicate sets of data are analyzed simultaneously with no common parameters, the global correlation of neighboring residuals is significant ($P < 0.005$) for five of the seven agonists tested; for every agonist, the global variance of residuals is significantly greater ($P < 0.02$) than that obtained with eq 3 ($n = 2$) (supplementary material Table III). The poor fit of the model to the present data differs from the good agreement reported recently by Ehlert (1985) for muscarinic receptors in homogenates of rabbit heart. In that investigation, however, the ratio of total G protein to total receptor was taken as 0.81. Considerations based on the work of Lee et al. (1986) and described above argue against an excess of receptors in preparations of hamster heart. Moreover, an analysis of the present data with $[G]_t/[R]_t$ fixed at 0.8 rather than 1.0 fails to provide a satisfactory fit. Four agonists yield a significant correlation of neighboring residuals ($P < 0.001$), and four yield a variance of residuals that exceeds that obtained with eq 3 ($P < 0.05$) (supplementary material Table III). Finally, we recently have studied the binding of carbachol in preparations of hamster heart following alkylation of 70% of the muscarinic sites with propylbenzilylcholine mustard; the data are not consistent with the notion that the sites of weakest affinity for agonists represent an excess of receptors over interacting G proteins in the native preparation.[3]

A reduction in $[G]_t/[R]_t$ from 1.0 to 0.8 does not lead to a satisfactory fit but nevertheless reduces the variance of residuals for all agonists tested in the present investigation (supplementary material Table III). Agreement between the ternary model and the data is markedly improved at values of this parameter that yield the best fit for each agonist: the global correlation of neighboring residuals is significant ($P < 0.05$) for only two agonists out of seven, and in every case, the global variance is lower than that obtained with eq 3 ($n = 2$) (supplementary material Table III). The estimates of $[G]_t/[R]_t$ vary widely, however, from 0.27 ± 0.01 with arecoline to 0.86 ± 0.02 with methacholine (supplementary material Table III). Differences in $[G]_t/[R]_t$ between pairs of agonists have been tested for significance according to the procedure used to compare values of F_2 (eq 3); among the 21 possible combinations (supplementary material Table IV), the differences are significant in 5 ($0.05 > P > 0.005$) and highly significant in 4 ($P < 0.001$). Since $[G]_t/[R]_t$ is expected to be independent of the agonist, the observed variation is inconsistent with the model.

A similar lack of agreement is found in the effect of GMP-PNP. At the concentrations used, the nucleotide (N) appears to be saturating with respect to its site on the G protein; in terms of the ternary model, this implies that all G is in the form GN. The interaction within the membrane therefore is between the receptor and GN rather than G, but the system is expected to behave quantitatively in the same manner. Agreement is poor, however, between best fits of the model and the binding patterns obtained for carbachol in the presence of GMP-PNP (supplementary material Table III). The global correlation of neighboring residuals from five experiments is highly significant irrespective of whether $[G]_t/[R]_t$ is fixed at 1.0 ($P < 0.00001$) or 0.8 ($P = 0.00001$), and the global variance greatly exceeds that obtained with eq 3 ($n = 2$) ($P < 0.00001$). The model closely approximates the data if $[G]_t/[R]_t$ is taken as a variable parameter (supplementary material Table III), but the value of 0.29 ± 0.02 is lower that that of 0.65 ± 0.03 obtained from five experiments in the absence of GMP-PNP. The difference is confirmed by the increase in the variance of residuals ($P < 0.00001$) when the data are fitted with a single value for all experiments rather than with two, separate values for experiments with and

[2] The mathematical representation of the ternary model used for these analyses differs from that described by Lee et al. (1986) in that both the radioligand and the competing agonist enter into the equations as total rather than free concentration. The numerical procedures utilized to compute [PR] and [PRG] will be described elsewhere. Depletion of the free ligand through binding to the receptor was negligible for agonists and generally was less than 5% for *N*-[^{3}H]methylscopolamine; the two expressions thus yield similar or identical values for all parameters. Values of K_A, K_{AG}, $[R]_t$ (or SA), and C were determined by regression; $[G]_t/[R]_t$ and K_G were dealt with as described in the text.

[3] M. A. Green and J. W. Wells, unpublished observations.

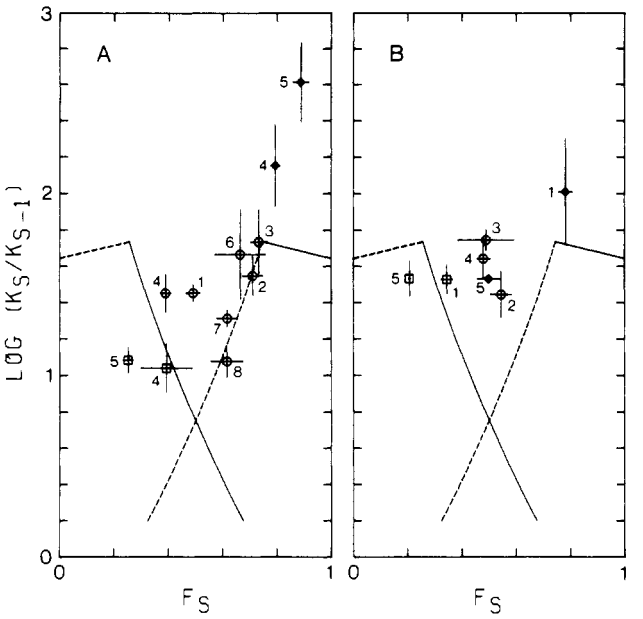

FIGURE 5: Comparison of parametric values derived from multisite analysis of experimental data and data simulated according to the ternary model. The points reflect parametric values derived from best fits of eq 3 to the data from replicate experiments with different agonists in homogenates of left ventricle (A) and left plus right atria (B). The number of classes of sites was taken as two (○) or three (◇, □) as described in the text; for those agonists that reveal three classes of sites, the data reflect the two classes of higher affinity [◇, log (K_2/K_1), $F_2/(F_1 + F_2)$] and the two classes of lower affinity [□, log (K_3/K_2), $F_3/(F_2 + F_3)$]. Values of log (K_S/K_{S-1}) and $F_S/(F_{S-1} + F_S)$ were calculated for individual experiments and averaged to obtain the mean and standard error plotted in the figure; mean values of the individual parameters are listed in Table I. The lines are taken from Figure 6 in the report by Lee et al. (1986) and indicate the limits imposed by the ternary model on values of log (K_S/K_{S-1}) and F_S. Individual agonists are as follows: 1, carbachol; 2, carbachol plus GMP-PNP; 3, arecoline; 4, oxotremorine; 5, methacholine; 6, oxotremorine M; 7, bethanechol; and 8, furmethide.

without the nucleotide. The addition of GMP-PNP also is accompanied by a change in the value of log K_A, which decreases from -5.07 ± 0.06 to -5.34 ± 0.02 at optimal values of $[G]_t/[R]_t$; there is a substantial increase in the variance ($P = 0.00064$) when a single value of log K_A is common to all experiments. The nucleotide is expected to alter K_G and possibly K_{AG} but to have no effect on either K_A or $[G]_t/[R]_t$.

The studies of Lee et al. (1986) allow parameters obtained from the multisite model (eq 3) or the Hill equation (eq 1) to be assessed for agreement with predictions of the ternary model. Such a comparison provides some insight into the discrepancies between the ternary model and the present data. For a ternary system with equal quantities of receptor and G protein, values of log (K_2/K_1) and F_2 (eq 3, $n = 2$) are expected to fall within the domains illustrated in Figure 5 (Lee et al., 1986). A comparison of the permitted domains with the parametric values summarized in Table I suggests that the model is at variance with the data for virtually all agonists studied in the present investigation. Among the agonists that reveal two classes of sites in the left ventricle (Figure 5A), the clearest discrepancy arises with carbachol. Parametric values from a large number of experiments are characterized by relatively low error, and the values of log (K_2/K_1) and F_2 are incompatible with the model irrespective of the preference of carbachol for R or for RG. Oxotremorine ($n = 2$) is only marginally out of bounds and appears to agree with a limiting case of the situation in which the agonist binds more tightly to RG than to R ($K_A/K_{AG} > 1$, $[R]_t/K_G < 0.01$). In contrast, the positions of furmethide, bethanechol, oxotremorine M, and arecoline all suggest that agonists bind more tightly to R than to RG ($K_A/K_{AG} < 1$).[4] Some agonists thus appear to promote formation of the RG complex, while others appear to promote its dissociation. Since the RG complex generally is believed to lie in the mechanistic pathway between the receptor and the cyclase, one expects that all agonists would have the same effect. Moreover, the four compounds that appear to promote net dissociation of the complex show a positive correlation between log (K_2/K_1) and F_2. Such an arrangement is opposite to the negative correlation predicted by the model and described by Lee et al. (1986). In atrial homogenates, arecoline and oxotremorine reveal two classes of sites, and both compounds yield values of log (K_2/K_1) and F_2 that lie outside the domains permitted by the model (Figure 5B).

The appearance of three classes of sites with some agonists implies an excess of receptors over G proteins (Lee et al., 1986). Such a possibility is not consistent with the present data, however, as noted above and illustrated further by the comparison in Figure 5. If it is assumed that the sites ostensibly of lowest affinity (K_3) represent uncoupled receptor, the value of log (K_2/K_1) exceeds the upper limit with both methacholine and oxotremorine in the left ventricle (Figure 5A). The discrepancy actually is greater than appears in the figure, since the permitted domains for that portion of the binding curve corresponding to potentially coupled receptors shrink in a manner similar to that found for the entire curve when $[G]_t$ exceeds $[R]_t$ [cf. supplementary material Figure 1 in Lee et al. (1986)]. Moreover, the corresponding values of $F_2/(F_1 + F_2)$ are sufficiently high to be compatible only with the situation in which free R is of higher affinity for agonists. If it is assumed that the sites of highest affinity (K_1) reflect uncoupled receptor, values of log (K_3/K_2) may be within the acceptable range, but the values of $F_3/(F_2 + F_3)$ imply that uncoupled receptor is of lower affinity for agonists. Similar discrepancies can be observed for the agonists that reveal three classes of sites in atrial preparations (Figure 5B).

The Hill coefficient for carbachol is increased upon the addition of GMP-PNP but remains significantly less than 1 in both atrial and ventricular homogenates. Other investigators have reported similar behavior for muscarinic receptors in cardiac membranes prepared from several species and according to various procedures (Hulme et al., 1980b; Rosenberger et al., 1980; Halvorsen & Nathanson, 1981; Harden et al., 1982; Burgisser et al., 1982; Waelbroeck et al., 1982; Hosey, 1983; Nathanson, 1983; Dunlap & Brown, 1984; Galper et al., 1984; Mattera et al., 1985). Analysis of the present data in terms of eq 3 indicates that the nucleotide acts to increase the relative number of sites ostensibly of lower affinity for carbachol. The binding patterns obtained at saturating concentrations of the nucleotide (N) are at variance with the ternary model on at least two counts. First, the values of log (K_2/K_1) and F_2 obtained for carbachol in ventricular homogenates suggest that the agonist favors R over RGN (Figure 5A), in contrast to the supposed preference for RG over R in the absence of nucleotide; in atrial homogenates, the values of log (K_2/K_1) and F_2 lie outside both of the domains shown in Figure 5. Second, the ternary model predicts a rightward shift in the position of the binding curve if GMP-PNP acts to weaken the affinity of G for R [cf. Figure 5 in

[4] When the ternary model was fitted to the present data, it was assumed that agonists bind more tightly to RG than to R ($K_{AG} < K_A$). Initial estimates of the various parameters were selected accordingly, and convergence is to a local minimum. A second minimum exists, however, that corresponds to the complementary arrangement in which agonists bind more tightly to R than to RG ($K_A < K_{AG}$).

Lee et al. (1986)]; whether the final curve reveals one or two classes of sites (eq 3) depends upon the value of $[R]_t/K_G$ in the presence of the nucleotide. The shift is expected to be substantial for carbachol in either the left ventricle or the atria, since Hill coefficients in the absence of nucleotide (Table I) approximate the lower limit of 0.67 that is possible with equal quantities of G protein and receptor. Values of K_S were unaffected by GMP-PNP in the present investigation (Table I and supplementary material Table II).

Reports of Hill coefficients that remain less than 1 in the presence of guanylyl nucleotides often are not accompanied by an analysis of the data in terms of multiple classes of sites. Indeed, parametric values derived from such models may be associated with considerable uncertainty when the data deviate only slightly from a rectangular hyperbola (De Lean et al., 1982). Where such an analysis has been attempted, however, the results tend to agree with those obtained with carbachol in the present investigation. Guanylyl nucleotides have been reported to alter F_S but to have little or no consistent effect on K_S in membrane preparations from rat heart (Hulme et al., 1980a,b), in homogenates of cultured cells from embryonic chick hearts (Galper et al., 1984), in membrane preparations from dog and guinea pig heart (Uchida et al., 1984), and in sarcolemmal preparations from canine ventricle (Mattera et al., 1985). Studies on the binding of radiolabeled agonists have revealed a similar effect: GMP-PNP has been shown to reduce capacity but not affinity for [^{3}H]oxotremorine M in washed membranes from rat heart (Harden et al., 1983) and for [^{3}H]acetylcholine in homogenates of rat hippocampus and striatum (Gurwitz et al., 1985).

Some investigators have reported that guanylyl nucleotides increase not only the value of F_2 but also the values of K_1 and K_2 for agonists in cardiac homogenates that appear to contain two classes of sites (Burgisser et al., 1982; Waelbroeck et al., 1982; Nathanson, 1983; Dunlap & Brown, 1984). The changes in affinity generally are small, however, and in some instances are not significant. They may derive in part from a concomitant increase in the affinity of the radiolabeled antagonist, although studies with radiolabeled oxotremorine M suggest that the nucleotide can alter the affinity of the agonist alone in some systems (Hulme et al., 1983). They also may reflect the resolution of the data and the choice of a two-site model; with the present data, for example, failure to recognize the need for a third site in the atria would result in an increase in both K_1 and K_2. In any event, reported changes in affinity are the same or similar at both classes of sites. In contrast, the ternary model predicts that the increase in K_1 will exceed the increase in K_2 for a system in which agonists promote the association of G protein and receptor. Moreover, reported values of log (K_2/K_1) and F_2 almost invariably imply that agonists bind with higher affinity to R than to RG (cf. Figure 5).

The foregoing considerations suggest that neither the multisite model (eq 3) nor the ternary model is consistent with the binding patterns obtained for agonists at cardiac muscarinic receptors. The physical significance of all parameters derived from either model therefore is uncertain, and parametric values ought to be interpreted with caution. Lee et al. (1986) have suggested, however, that estimates of K_S derived from the multisite model seem to reflect the equilibrium dissociation constants of agonists for different forms or states of the receptors. If so, uncertainty over the mechanism underlying the behavior of agonists relates primarily to the observation that the value of F_S depends upon the agonist. It follows that an understanding of the binding patterns is dependent, at least in part, upon a resolution of the paradox wherein individual agonists discern multiple classes of apparently noninteracting sites while simultaneously controlling the relative number of sites in one or other classes.

Acknowledgments

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Supplementary Material Available

One figure depicting the binding of *N*-[^{3}H]methylscopolamine in the absence and presence of GMP-PNP, two tables of statistical data derived from analyses in terms of eq 3, and two tables of parametric values and statistical data derived from analyses in terms of the ternary model (7 pages). Ordering information is given on any current masthead page.

Registry No. GMP-PNP, 34273-04-6; carbachol, 51-83-2; methacholine, 55-92-5; oxotremorine, 70-22-4; arecoline, 63-75-2; furmethide, 541-64-0.

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