Cardiac Muscarinic Receptors. Relationship between the G Protein and Multiple States of Affinity†

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ABSTRACT: An expanded version of the mobile receptor model has been assessed in studies on the binding of *N*-[^{3}H]methylscopolamine and [^{35}S]GTPγS to cardiac muscarinic receptors and their attendant G proteins in ventricular membranes from hamster. The model comprises two pools of receptor, one of which lacks G proteins, and a heterogeneous population of G proteins that compete for the receptor within the G protein-containing pool. To guide the formulation of the model itself and to define the various parameters, data were combined from assays performed under various conditions with native membranes and following irreversible blockade of about 80% of the receptors with propylbenzilylcholine mustard. Multiple G proteins are indicated primarily by multiple states of affinity evident in the dose-dependent effect of guanyl nucleotides on the binding of carbachol; G protein-free receptors are indicated by sites of low affinity for carbachol that survive treatment with the mustard. The expanded model generally succeeds where more frugal schemes have been inadequate, but it nevertheless fails to yield a mechanistically consistent description of the data. Guanyl nucleotides and partial alkylation do not affect the inhibitory potency of carbachol in a manner consistent with their supposed effect on the equilibrium between uncoupled and G protein-coupled receptors. As inferred from the model, G proteins are lost upon alkylation of the receptor, and their numbers are regulated by guanyl nucleotides. Parameters estimated via *N*-[^{3}H]methylscopolamine are wholly inconsistent with the same parameters estimated via [^{35}S]GTPγS. The failure of the model suggests that multiple states of affinity may not arise from a ligand-regulated equilibrium between free receptors and G proteins on the one hand and one or more RG complexes on the other.

Muscarinic and other G protein-linked receptors have been reported to promote the exchange of triphosphonucleosides for GDP at more than a stoichiometric equivalent of G proteins (Birnbaumer *et al.*, 1990). That amplification is commonly held to reflect the successive formation and dissociation of transient complexes such that the receptor can act catalytically. Mechanistic proposals thus tend to be based upon the notion of turnover or exchange at the level of the RG complex. This view is consistent with the dependence of G protein-mediated responses on the local concentration of receptors, which recalls the pattern expected if the former were determined by the integrated output of the latter (Stephenson, 1956; Furchgott, 1966). In erythrocyte membranes, for example, partial alkylation of β-adrenergic receptors has been shown to slow the rates of activation and deactivation of adenylate cyclase by agonists but not their maximal effect (Tolkovsky and Levitzki, 1978; Arad *et al.*, 1981). In cultured L cells, both the maximal response of adenylate cyclase and the potency of epinephrine were found to vary with the level of expression of β_2-adrenergic receptors (Whaley *et al.*, 1994). In CHO cells, the number of m2 muscarinic receptors determined the potency of agonists and their maximal inhibitory effect on adenylate cyclase (Vogel *et al.*, 1995).

The binding of agonists to muscarinic and other G protein-linked receptors reveals a dispersion of affinities that reflects, at least in part, the influence of the G protein (Hulme *et al.*, 1990). Moreover, quantitative measures of the dispersion or its sensitivity to guanyl nucleotides correlate with pharmacological properties such as efficacy and intrinsic activity [*e.g.*, Birdsall *et al.* (1977), Kent *et al.* (1980), Ehlert (1985), and Potter and Ferrendelli (1989)]. The binding patterns thus appear to be a manifestation of the mechanistic events that culminate in a response, and it is widely accepted that sites of low and high affinity for agonists represent coexisting populations of uncoupled and G protein-coupled receptors.

A dispersion of affinities *per se* is inherently ambiguous. When there is only one independent variable, typically the concentration of an agonist, it is not possible to distinguish among different mechanistic schemes that predict Hill coefficients <1 for systems at equilibrium. The notion of a ligand-regulated, transient complex between receptor and G protein therefore derives primarily from other data. Particularly striking is the 500-fold amplification reported for the rhodopsin-catalyzed turnover of guanyl nucleotides at the GTP-specific site of transducin (Fung and Stryer, 1980). Also, the apparent size of α-adrenergic, β-adrenergic, and D_2-dopaminergic receptors is reported to be larger when the membranes are treated with an agonist prior to solubilization (Limbird *et al.*, 1980; Smith and Limbird, 1981; Kilpatrick and Caron, 1983), and agonists increase the tendency of G_o and G_i to remain associated with immunoprecipitated mus-

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carinic receptors (Matesic *et al.*, 1991). In contrast, GMP-PNP has been found to reduce the apparent size of M_2 muscarinic receptors (Berrie *et al.*, 1984b), and the agonist-stimulated GTPase activity of affinity-purified D_2 receptors was less if the column was eluted with GMP-PNP prior to elution with haloperidol (Senogles *et al.*, 1987). Such observations are consistent with the notion that the receptor−G protein complex is stabilized by agonists and destabilized by guanyl nucleotides, at least in solution.

The "ternary complex" or "mobile receptor" model constitutes an explicit description of the interaction between receptor and G protein (De Lean *et al.*, 1980). Coupled and uncoupled receptors are thought to coexist in equilibrium, at least under the conditions of a binding assay; antagonists and agonists shift the system toward one form or the other, depending upon their relative affinity for the two states. The model thereby predicts a dispersion of affinities whenever total R equals or exceeds total G; moreover, it suggests a mechanism for the allosteric effects of guanyl nucleotides, which would perturb the equilibrium through their differential affinity for free and receptor-coupled G proteins.

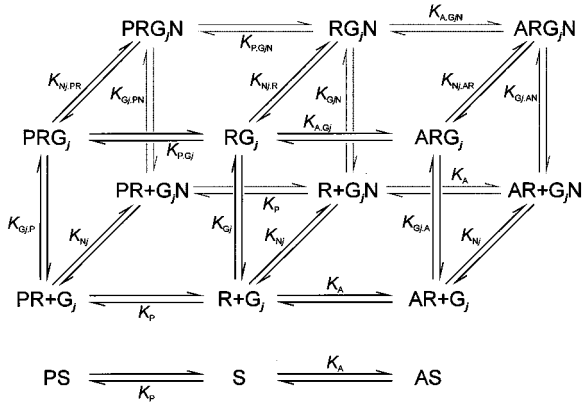
Quantitative applications of the mobile receptor model are infrequent, but it has been reported to describe the binding of agonists to β-adrenergic receptors (De Lean *et al.*, 1980), D_2-dopaminergic receptors (Wreggett and De Lean, 1984), A_1 adenosine receptors (Leung *et al.*, 1990), α_2-adrenergic receptors (Neubig *et al.*, 1988), and M_2-muscarinic receptors (Ehlert, 1985, 1987; Ehlert and Rathbun, 1990; Minton and Sokolovsky, 1990). Success has been mixed, as illustrated by the common implication that guanyl nucleotides such as GMP-PNP[1] cause an irreversible loss of G proteins from the system. Owing to this and other anomalies, the model has not provided a mechanistically consistent description of the binding patterns and the changes effected by guanyl nucleotides (Wong *et al.*, 1986; Lee *et al.*, 1986).

The present paper describes an extended version of the mobile receptor model, which has been examined for its ability to account for the binding properties of muscarinic receptors in myocardial membranes. Agonists, guanyl nucleotides, and the irreversible antagonist propylbenzilylcholine mustard have been used in concert with *N*-[^{3}H]-methylscopolamine and [^{35}S]GTPγS under various conditions to define the parameters of the model and to test for internal consistency. Despite extensions that overcome the deficiencies of earlier schemes, the model is found to be unacceptable on several counts. In the accompanying paper, the data have been examined in terms of the alternative notion of cooperativity among interacting sites (Chidiac *et al.*, 1997).

MATERIALS AND METHODS

Experimental Procedures. All data were obtained as described in the accompanying paper (Chidiac *et al.*, 1997). *N*-[^{3}H]Methylscopolamine chloride was purchased from New England Nuclear (85.0 Ci/mmol) and Amersham (74.0 Ci/mmol), and [^{35}S]GTPγS was purchased from New England Nuclear (1100−1325 Ci/mmol). Ventricular membranes were prepared from the hearts of Syrian golden hamsters and were washed extensively (5.0 mM HEPES, 1.0 mM EDTA, 200 μg/mL bacitracin, pH 8.0) to remove endogenous ligands, particularly GDP. Assays were performed on the native membranes and, in some experiments with *N*-[^{3}H]-methylscopolamine, on membranes in which most of the receptors had been inactivated by the irreversible antagonist propylbenzilylcholine mustard. Binding was measured in two buffers selected as optimal for studies with *N*-[^{3}H]-methylscopolamine on the one hand (buffer A, 10 mM HEPES, 1.0 mM $MgCl_2$, pH 7.45) and [^{35}S]GTPγS on the other (buffer B, 10 mM HEPES, 5.0 mM $MgCl_2$, 100 mM NaCl, 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, pH 7.40). For comparisions between *N*-[^{3}H]methylscopolamine and [^{35}S]GTPγS, the former also was measured in buffer B. The concentration of protein was 0.67 g/L (buffer A) or 0.5 g/L (buffer B), and the reaction mixture was equilibrated at 30 °C for 45 min (buffer A) or 2.5 h (buffer B). Bound and free radioligand were separated by microcentrifugation.

Scheme 1



Analysis of Data. Total observed binding was taken as the dependent variable throughout (B_{obsd}, dpm/mL). The expression fitted to the data was eq 1, in which $[P]_b$ represents the specific binding of the radioligand at a total concentration $[P]_t$; SA is the specific radioactivity (Ci/mmol), and NS is the fraction of unbound radioligand that appears as nonspecific binding.

$$B_{obsd} = \{[P]_b + (NS)([P]_t - [P]_b)\}(SA)(2.22 \times 10^{12}) \quad (1)$$

The value of $[P]_b$ in eq 1 was calculated according to Scheme 1, which comprises two forms of the receptor (R, S) and a potentially heterogeneous population of G proteins (G_j, where $j = 1, 2, ..., n$). Each R can interact with any G_j within the relevant compartment of the membrane to form the spontaneously dissociating complex RG_j, as envisaged in the ternary complex model described by De Lean *et al.* (1980). It follows that all R and G_j are colocalized within a single compartment or within multiple compartments that are indistinguishable in the binding assays. There is no interaction between S and G_j, perhaps owing to their physical separation within the membrane, and there is no interconversion between R and S. Radiolabeled and unlabeled ligands that compete for the receptor are shown as P and A, respectively, and the guanyl nucleotide is shown as N. Unbound ligands have been omitted from the figure, but the various equilibria are identified by the corresponding dissociation constants (*e.g.*, $K_A = [A][R]/[AR]$, $K_{A.Gj} = [A][RG_j]/[ARG_j]$, $K_{A.GjN} = [A][RG_jN]/[ARG_jN]$, $K_{Gj} = [R][G_j]/[RG_j]$, $K_{Gj.A} = [AR][G_j]/[ARG_j]$, $K_{Gj.AN} = [AR][G_jN]/[ARG_jN]$, $K_{Nj} = [G_j][N]/[G_jN]$, etc).

[1] Abbreviations: GMP-PNP, guanylylimidodiphosphate; GTPγS, guanosine 5′-*O*-[3-(γ-thio)triphosphate]; HEPES, sodium *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; NMS, *N*-methylscopolamine.

Total specific binding was calculated according to eq 2. The concentrations of radioligand-containing complexes and of other species discussed in the text were calculated from the equilibrium dissociation constants and the free concentrations of the reactants. The latter were obtained by solving a set of implicit equations derived from the equations of state for all ligands, receptors, and G proteins in the system. Solutions were obtained according to the Newton−Raphson procedure, but successful convergence often required initial estimates that were close to the desired roots. Values near or equal to the answer were computed in an iterative procedure that involved successive estimates of the free concentrations of all proteins on the one hand (*i.e.*, [R], [S], and [G_j]) and of all ligands on the other (*i.e.*, [P], [A], and [N]). The total concentrations of all ligands were taken as equal to the free concentrations and substituted in explicit expressions to obtain the free concentrations of receptors and G proteins; the latter then were used to obtained revised estimates of the former, and the cycle was repeated until convergence occurred. Further details regarding the formulation of Scheme 1 have been described elsewhere (Wells, 1992).

$$[P]_b = [PS] + [PR] + \sum_{j=1}^{n} [PRG_j] + \sum_{j=1}^{n} [PRG_jN] \quad (2)$$

Parameters entered explicitly into the fitting procedure are K_A, $K_{A.Gj}$, $K_{A.GjN}$, K_P, $K_{P.Gj}$, $K_{P.GjN}$, K_{Nj}, K_{Gj}, $K_{Gj.N}$, $[R]_t + [S]_t$, $[S]_t/([R]_t + [S]_t)$, $[G_j]_t/[R]_t$, and NS. In some analyses of pooled data from native and alkylated membranes, it proved useful to express $[G_j]_t/[R]_t$ for the latter in terms of that for the former (*i.e.*, $[G_j]_t/[R]_{t,alk} = a[G_j]_t/[R]_{t,nat}$). Also, the ratio $[G_j]_t/[R]_t$ for one G protein sometimes was expressed relative to that for another (*i.e.*, $[G_2]_t/[R]_t = b[G_1]_t/[R]_t$). Other parameters are self-evident in terms of the above (*e.g.*, $[R]_t$, $[S]_t$, $[G_j]_t$) or implicit in the multiple closed loops of Scheme 1 (*e.g.*, $K_A/K_{A.Gj} = K_{Gj}/K_{Gj.A}$), and the values were calculated as required.

The concentrations of all reactants in Scheme 1 are expressed as moles per liter of homogenate. Such units are notional for the interaction between R and G_j, which presumably occurs within the plane of the membrane. Simultaneous analyses therefore were restricted to data acquired under essentially the same conditions, including the same concentration of protein. Within that restriction, however, differences in $[R]_t$ and $[G_j]_t$ among data from different experiments are assumed to denote differences in the local concentration within the membrane.

Data on the binding of *N*-[^{3}H]methylscopolamine were analyzed in terms of Scheme 1 taken as illustrated above. Since the model is shown with only one ligand to the G protein, data on the binding of [^{35}S]GTPγS require an extension to accommodate the competitive effects of unlabeled guanyl nucleotides (*e.g.*, Figure 4). Multiple classes of G protein proved redundant with [^{35}S]GTPγS, however, and the data were well described by assuming a single class of G protein in the receptor-accessible pool. Scheme 1 therefore can be applied directly to the data acquired with [^{35}S]GTPγS if *n* is taken as 1 and the symbols for the receptor and G protein are interchanged.

All analyses involved multiple sets of data, and parameters were allocated among the different sets as described in the text and in the footnotes to the tables. For most parameters, the value was defined unambiguously by a clear minimum in the weighted sum of squares. When the sum of squares was asymptotic in one direction or the other, the value of the parameter was mapped to determine the upper or lower bound consistent with the data. In such cases, the value was fixed appropriately during subsequent analyses. Parametric values derived from a single analysis are presented together with the error as estimated from the diagonal elements of the covariance matrix. When two or more estimates were averaged to obtain the mean, the value is presented together with the standard error. Further details regarding the analyses and related statistical procedures are described in the accompanying paper (Chidiac *et al.*, 1997).

At various places in the text, the results of the present analyses are compared with those obtained when the receptors are depicted as a mixture of distinct, noninterconverting, and noninteracting sites (*i.e.*, the multisite model). The latter approach is described in the accompanying paper (Chidiac *et al.*, 1997), where the model is identified as Scheme 1. The equilibrium dissociation constants of the radioligand (P) and the unlabeled ligand (A) for the sites of type *j* (R_j) are designated K_{Pj} and K_{Aj}, respectively, and F_j is the corresponding fraction of all sites represented by R_j. Different classes of sites are numbered in order of decreasing affinity for the unlabeled ligand (*i.e.*, $K_{A1} < K_{A2} < K_{A3}$).

RESULTS

Binding of N-[^{3}H]Methylscopolamine. The binding patterns obtained for *N*-[^{3}H]methylscopolamine at low ionic strength revealed multiple states of affinity (*e.g.*, $n_H < 1$) and a leftward shift in the presence of GMP-PNP (Chidiac *et al.*, 1997). In the context of the mobile receptor model, multiple states imply that the radioligand differs in affinity for R and RG (*i.e.*, $K_P \neq K_{P.G}$); the reduction in EC_{50} implies that the state of higher affinity is favored by the nucleotide. Preliminary analyses indicated that one class of G protein is sufficient for Scheme 1 to describe the data obtained at graded concentrations of *N*-[^{3}H]methylscopolamine, which was found to bind more tightly to R than to RG or RGN (*i.e.*, $K_P < K_{P.G}$, $K_P < K_{P.GN}$). The parameter K_P is well-defined by the data, but only lower limits can be placed on $K_{P.G}$ and $K_{P.GN}$. Values above about 10 nM are without effect on the weighted sum of squares (curves a and b in Figure 1) or on the values of other parameters. Lower values increase the sum of squares, which significantly exceeds the asymptotic level when $K_{P.G}$ is less than about 0.23 nM (log $K_{P.G} = -9.64$, curves a, no GMP-PNP) or when $K_{P.GN}$ is less than 0.14 nM (log $K_{P.GN} = -9.84$, curves b, with GMP-PNP).

The goodness of fit in effect is compromised at any value of $K_{P.G}$ or $K_{P.GN}$ that implies appreciable levels of PRG or PRGN. Hill coefficients <1 derive from values of K_G or $K_{G.N}$ such that an appreciable fraction of the receptors in the G-containing compartment are associated with G protein in the absence of *N*-[^{3}H]methylscopolamine. The antagonist reduces the affinity of R for G or GN (*i.e.*, $K_P/K_{P.G} = K_G/K_{G.P}$), and the effect is sufficient to preclude coupling.

Antagonist-promoted destabilization of the RG complex also emerges when the binding properties of *N*-[^{3}H]methylscopolamine at low ionic strength are inferred from its effect on the inhibitory behavior of carbachol [*i.e.*, Figure 1 in Chidiac *et al.* (1997)]. Data acquired at graded concentra-

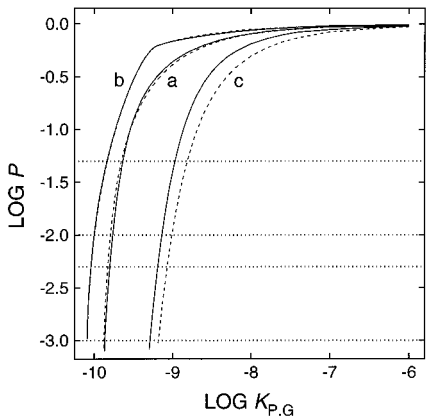


FIGURE 1: Effect of $K_{P.G}$ on the goodness of fit obtained with Scheme 1. Equation 1 was fitted to data acquired at graded concentrations of [^{3}H]NMS (a, no GMP-PNP; b, 0.1 mM GMP-PNP) or at 0.04 and 1.0 nM *N*-[^{3}H]methylscopolamine and graded concentrations of carbachol (c, no GMP-PNP). The population of G proteins was assumed to be homogeneous (eq 2, $n = 1$). The parameter $[S]_t/([R]_t + [S]_t)$ either was optimized (—) or was fixed at zero (- - -), and $K_{P.G}$ was fixed at different values from <0.1 nM to 1 mM. The weighted sum of squares from the analysis at each value of $K_{P.G}$ was compared with the asymptotic minimum to obtain the *F* statistic and the corresponding value of log *P* plotted on the ordinate. Each relationship shown in the figure reflects six sets of data from three independent experiments; binding at graded concentrations of the radioligand was measured concurrently in the absence and presence of 0.01 mM unlabeled NMS (a, b), and binding at graded concentrations of carbachol was measured concurrently at both concentrations of the radioligand (c) . In each analysis, single values of K_P, $K_{P.G}$, $[R]_t/[G]_t$, and $[S]_t/([R]_t + [S]_t)$ were common to all six sets of data. The horizontal dotted lines indicate the 95%, 99%, 99.5%, and 99.9% levels of confidence. Values of log $K_{P.G}$ corresponding to 95% confidence are as follows for variable and fixed values of $[S]_t/([R]_t + [S]_t)$, respectively: a, −9.64 and −9.65; b, −9.84 and −9.83; c, −8.97 and −8.82.

tions of the agonist and two concentrations of the radioligand yield a profile for $K_{P.G}$ similar to that observed with graded concentrations of the radioligand (Figure 1, curves c). The sum of squares is minimal at high values of $K_{P.G}$ and increases at lower values, the increase becoming significant when $K_{P.G} = 1.1-1.5$ nM ($P < 0.05$). Data from both experimental protocols thus imply that $K_{P.G}$ exceeds K_P sufficiently to preclude appreciable levels of G protein-coupled receptor in the presence of *N*-methylscopolamine.

Binding of *N*-[^{3}H]methylscopolamine at high ionic strength was insensitive to GMP-PNP and revealed a Hill coefficient of 1 (Chidiac *et al.*, 1997). The data therefore are ambiguous: the radioligand may bind to R and RG with equal affinity (*i.e.*, $K_P = K_{P.G}$); alternatively, the radioligand may favor R over RG (*i.e.*, $K_P < K_{P.G}$), with little or no coupling of G and unliganded R (*i.e.*, $K_G \gg [R]_t$ or $[G]_t$). The latter possibility is consistent with the pattern of binding at low ionic strength; also, data presented below suggest that the value of K_G is indeed smaller in buffer A than in buffer B.

Since $K_{P.G}$ and $K_{P.GN}$ are defined only by a lower bound, the value of each has been fixed at 10 μM in all subsequent calculations. Despite this restriction, data acquired only at graded concentrations of the radioligand are insufficient to yield a unique solution in terms of the model. The remaining parameters can be strongly correlated, most notably in the case of $[G]_t/[R]_t$ and $[S]_t/([R]_t + [S]_t)$, and there is no clear minimum in the sum of squares. The problem is less severe when the data obtained with and without GMP-PNP at low ionic strength are analyzed in concert: since the nucleotide ought to be without effect on K_P, $[G]_t/[R]_t$, or $[S]_t/([R]_t + [S]_t)$, the number of parameters can be reduced accordingly. This constraint is accompanied by an increase in the sum of squares ($P = 0.0020$), perhaps signaling a problem with the model, but the fitted curves are in good agreement with the data. The parametric values are listed in Table 1A. GMP-PNP effected a 3.3-fold reduction in the affinity of G for R, in accord with the notion that guanyl nucleotides promote uncoupling of the RG complex.

All parameters are better defined by the data obtained at graded concentrations of carbachol and two concentrations of *N*-[^{3}H]methylscopolamine. The fitted curves are in excellent agreement with the data, and the parametric values are listed in Table 1B. There is a significant increase in the global sum of squares when $[S]_t/([R]_t + [S]_t)$ is fixed at zero ($P = 0.00022$), suggesting that an appreciable number of the labeled sites are inaccessible to G proteins. A similar distinction is not possible with the relatively featureless data obtained at graded concentrations of the radioligand (Table 1A).

Effects of Carbachol, GMP-PNP, and the Local Concentration of Receptors on the Binding of N-[^{3}H]Methylscopolamine at Low Ionic Strength. Cardiac muscarinic receptors have been shown to associate with G_i and G_o (Matesic *et al.*, 1991; Wreggett and Wells, 1995); moreover, the complex effects of GMP-PNP, GTPγS, and GDP on the binding of carbachol suggest that the nucleotide-specific sites are not homogeneous [Figure 2 in Chidiac *et al.* (1997)]. Preliminary analyses in terms of Scheme 1 confirmed that a single class of G proteins is insufficient to describe some combinations of data. The fit is better with two classes, at least in some respects, but each additional class of G proteins adds six parameters to the model (*i.e.*, $K_{A.Gj}$, $K_{A.GjN}$, K_{Nj}, K_{Gj}, $K_{Gj.N}$, $[G_j]_t/[R]_t$). The problem is severely underdetermined with only one independent variable, and a unique solution requires data from several experiments.

Most of the parameters are defined by the combination of data illustrated in Figure 2. Binding to native membranes was characterized at graded concentrations of *N*-[^{3}H]methylscopolamine, carbachol, and GMP-PNP in various combinations. Also included are data from membranes pretreated with sufficient propylbenzilylcholine mustard to reduce the capacity for *N*-[^{3}H]methylscopolamine by about 82%. The concentration of functional receptors was adjusted to perturb the effect of carbachol on the supposed equilibria between R and G_j. The solid lines in Figure 2 illustrate the best fit of Scheme 1 ($n = 2$), and the corresponding parametric values are listed in Tables 2 and 3. Mechanistic consistency was enforced through the assignment of parameters and related constraints, except as described below for the effect of the mustard on $[G_j]_t/[R]_t$. According to the model, 12−18% of the receptors were inaccessible to G proteins ($[S]_t/([R]_t + [S]_t)$, Table 3). That value is narrowly defined for each preparation, and the fit is severely compromised ($P < 0.00001$) if it is assumed that all receptors were equally accessible (*i.e.*, $[S]_t/([R]_t + [S]_t) = 0$).

Scheme 1 provides a first approximation of the data, but systematic deviations are evident in the binding of carbachol. Even with two classes of G protein, the model cannot accommodate the high-affinity sites that persist in native membranes at saturating concentrations of GMP-PNP (Figure 2A). The discrepancy derives from the nucleotide-induced decrease in the affinity of the receptor for G_1 (*cf.* log K_{G1}

Table 1: Direct and Inferred Binding of *N*-[^{3}H]Methylscopolamine in Terms of Scheme 1 ($n = 1$)[a]

analysis	variable ligand	GMP-PNP (mM)	$-\log K_P$	$-\log K_G$	$[R]_t + [S]_t$ (pM)	$[S]_t/([R]_t + [S]_t)$	$[G]_t/[R]_t$
A	[^{3}H]NMS	0	10.31 ± 0.08	10.63 ± 0.52	63 ± 6	0.054[b]	1.0[b]
		0.1		10.11 ± 0.46	63 ± 6		
B	carbachol, [^{3}H]NMS	0.0	10.04 ± 0.04	10.63 ± 0.17	70 ± 4	0.081 ± 0.40	1.0 ± 0.5

[a] Equation 1 was fitted to estimates of total binding at graded concentrations of [^{3}H]NMS (A) or carbachol (B). The former was measured concomitantly with the radioligand alone, the radioligand plus 0.1 mM GMP-PNP, and the radioligand plus 0.01 mM unlabeled NMS; the latter was measured concomitantly at two concentrations of the radioligand (0.04 and 1 nM). Each experiment was performed three times, and the data were combined for simultaneous analyses. The parameters were assigned as follows. A, Single values of K_P, $[G]_t/[R]_t$, and $[S]_t/([R]_t + [S]_t)$ were common to all of the data; single values of K_G were common to data acquired either with or without GMP-PNP. B, Single values of K_A, $K_{A.G}$, K_P, K_G, $[G]_t/[R]_t$, and $[S]_t/([R]_t + [S]_t)$ were common to all of the data. The fitted values of log K_A and log $K_{A.G}$ are -4.99 ± 0.43 and -7.93 ± 0.10, respectively. The quantity $[R]_t + [S]_t$ was estimated individually for each experiment, and the three values were averaged to obtain the mean ($\pm$SEM) listed in the table. [b] $[G]_t/[R]_t$ and $[S]_t/([R]_t + [S]_t)$ are strongly correlated, and the latter is indistinguishable from zero.

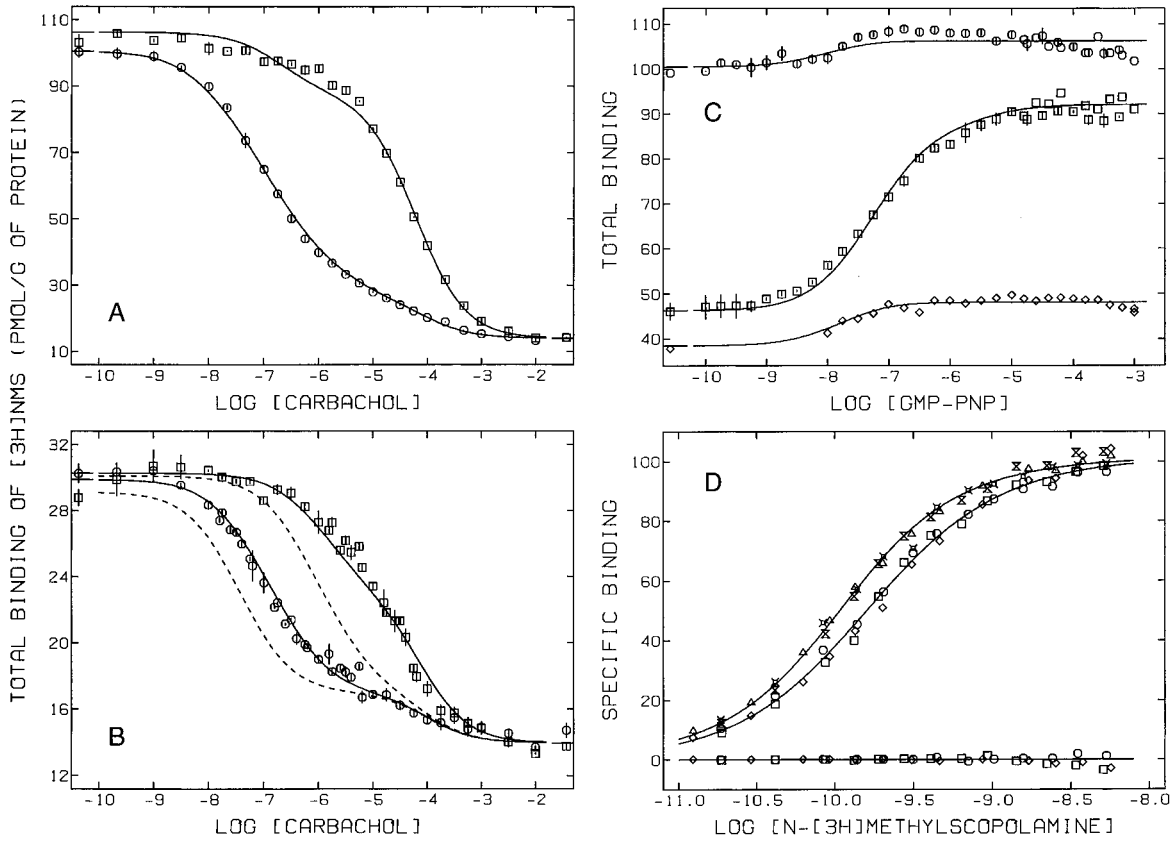


FIGURE 2: Fit of Scheme 1 to the binding of *N*-[^{3}H]methylscopolamine, carbachol, and GMP-PNP in preparations of native and alkylated membranes at low ionic strength. Total binding was measured following equilibration of the membranes in buffer A at graded concentrations of the ligand shown on the abscissa. Each panel contains the combined data from three experiments, except as shown below in brackets, and the conditions were as follows: (A) native membranes and 0.96−1.00 nM [^{3}H]NMS, no GMP-PNP (○), 0.1 mM GMP-PNP (□); (B) alkylated membranes and 0.59−1.03 nM [^{3}H]NMS, no GMP-PNP (○), 0.1 mM GMP-PNP (□); (C) native membranes and 0.98−1.03 nM [^{3}H]NMS, no carbachol (○), 0.56−1.0 μM carbachol [3] (□); native membranes and 98 pM [^{3}H]NMS [1] (◇); (D) [^{3}H]NMS plus 0.1 mM GMP-PNP (⊗, △, hourglass; leftward curve), [^{3}H]NMS alone (○, ◇, □; rightward curve), [^{3}H]NMS plus 0.01 mM unlabeled NMS (baseline). Different symbols in panel D denote data from different experiments (○, ⊗; ◇, △; □, hourglass). The solid lines represent the best fit of the model ($n = 2$) to the data taken together, and the parametric values are listed in Tables 2 and 3. The dashed lines in panel B were computed for alkylated membranes with the values of $[G_1]_t$ and $[G_2]_t$ taken as equal to those in native membranes (*i.e.*, $[R]_t + [S]_t = 11.76$, $[S]_t/([R]_t + [S]_t) = 0.1847$, $[G_1]_t/[R]_t = 4.969$, $[G_2]_t/[G_1]_t = 0.3076$); other parameters were as listed in Table 2. The left and right curves represent binding in the absence of guanyl nucleotide and in the presence of 0.1 mM GMP-PNP, respectively. Values plotted on the ordinate were obtained according to eq 6 in Chidiac *et al.* (1997); the mean value of $[P]_t$ used for panels A−C is 1.00 ± 0.01 nM, and the concentration of carbachol used for the middle curve in panel C is 0.56 μM. Points shown at the lower and upper ends of the abscissa indicate binding in the absence of unlabeled ligand (A−C) and in the presence of 0.01 mM unlabeled NMS (A, B), respectively.

and log $K_{G1.N}$ in Table 2), which represents 76% of all G proteins in the system (Table 3). Such a change is expected to weaken the inhibitory potency of agonists in the manner illustrated by the fitted curves in Figure 2A. As described previously (Lee *et al.*, 1986), the effect would appear as an increase in the value of K_{A1} when the data are analyzed in terms of distinct and independent sites. In contrast, GMP-PNP was without effect on K_{A1} with any of the agonists tested [Table 2 in Chidiac *et al.* (1997)].

A further discrepancy emerges from the binding of carbachol to alkylated membranes. The fitted curves in Figure 2B were obtained with no constraint on the ratio of G proteins to receptors (*i.e.*, $([G_1]_t + [G_2]_t)/[R]_t$). Since the ratio is seen to increase from about 1 in the native membranes

Table 2: Affinities Inferred from the Binding of *N*-[^{3}H]Methylscopolamine at Low Ionic Strength: Analysis in Terms of Scheme 1 ($n = 2$)[a]

reactants	parameter	value
cabachol (A) + R	log K_A	-5.23 ± 0.06
cabachol (A) + RG_1	log $K_{A.G1}$	-8.03 ± 0.05
cabachol (A) + RG_2	log $K_{A.G2}$	-9.26 ± 0.31
cabachol (A) + RG_1N	log $K_{A.G1N}$	<-6.0[b]
cabachol (A) + RG_2N	log $K_{A.G2N}$	-7.83 ± 0.20
[^{3}H]NMS (P) + R	log K_P	-10.20 ± 0.03
GMP-PNP (N) + G_1	log K_{N1}	-8.09 ± 0.04
GMP-PNP (N) + G_2	log K_{N2}	-6.63 ± 0.22
receptor (R) + G_1	log K_{G1}	-10.49 ± 0.05
receptor (R) + G_2	log K_{G2}	-10.23 ± 0.32
receptor (R) + G_1N	log $K_{G1.N}$	>-9.5[b]
receptor (R) + G_2N	log $K_{G2.N}$	-10.37 ± 0.24

[a] Equation 1 was fitted to estimates of total binding in preparations of native and alkylated membranes. The analysis involved 28 sets of data from 13 experiments in which [^{3}H]NMS, carbachol, or GMP-PNP was taken as the variable ligand. The conditions in preparations of native membranes were as follows, with the number of repeats for each type of experiment shown in parentheses: graded concentrations of the radioligand alone, plus 0.1 mM GMP-PNP, and plus 0.01 mM unlabeled NMS (3) (Figure 2D); 0.96−1.00 nM [^{3}H]NMS and graded concentrations of carbachol, alone and together with 0.1 mM GMP-PNP (3) (Figure 2A); 0.98−1.03 nM [^{3}H]NMS and graded concentrations of GMP-PNP, alone and together with 1.0 or 0.56 μM carbachol (3) (Figure 2C); 97 pM [^{3}H]NMS and graded concentrations of GMP-PNP (1) (Figure 2C). The alkylated preparation was assayed at 0.59−1.03 nM [^{3}H]NMS and graded concentrations of carbachol, alone and together with 0.1 mM GMP-PNP (3) (Figure 2B). Except as described in the text, parameters were assigned to force internal consistency in terms of the model. Estimates listed in the table are fitted values common to all of the data except in the case of K_A, $K_{A.Gj}$, and $K_{A.GjN}$, which are not relevant to binding at graded concentrations of the radioligand. The corresponding estimates of capacity are listed in Table 3. The analysis was constrained to preclude solutions in which the binding of [^{3}H]NMS decreased at concentrations of GMP-PNP above 0.32 μM; such effects were small and inconsistent over the course of the investigation. [b] The values of log $K_{A.G1N}$ and log $K_{G1.N}$ are negatively correlated such that a change in one can compensate almost fully for a change in the other. Neither is defined uniquely by the data, but the sum of squares is increased at values outside the limits shown in the table ($P < 0.05$) (*cf.* Figure 1). Other parameters are independent of $K_{A.G1N}$ and $K_{G1.N}$ within those limits. The values listed here and in Table 3 were obtained with log $K_{G1.N}$ fixed at -6.0, and the corresponding value of log $K_{A.G1N}$ is -9.49 ± 0.15.

Table 3: Capacities Inferred from the Binding of *N*-[^{3}H]Methylscopolamine at Low Ionic Strength: Analysis in Terms of Scheme 1 ($n = 2$)[a]

	preparation	
parameter[b]	native	alkylated
$[R]_t + [S]_t$[c] (pM)	67 ± 3	12 ± 1
$[S]_t/([R]_t + [S]_t)$	0.12 ± 0.01	0.18 ± 0.02
$[G_2]_t/[G_1]_t$	0.31 ± 0.02	
$[G_1]_t/[R]_t$	0.80 ± 0.04	1.9 ± 0.2
$[G_2]_t/[R]_t$	0.25	0.57
$([G_1]_t + [G_2]_t)/[R]_t$	1.05	2.44
$[R]_t$ (pM)	60	9.6
$[S]_t$ (pM)	7.9	2.2
$[G_1]_t$ (pM)	48	18
$[G_2]_t$ (pM)	15	5.5
$[G_1]_t + [G_2]_t$ (pM)	62	23

[a] Equation 1 was fitted to estimates of total binding in preparations of native and alkylated membranes, as described in the footnotes to Table 2. Capacities were estimated in terms of the parameters $[R]_t + [S]_t$, $[G_1]_t/[R]_t$, $[G_2]_t/[G_1]_t$, and $[S]_t/([R]_t + [S]_t)$. A single value of $[G_2]_t/[G_1]_t$ was common to all of the data, and separate values of $[G_1]_t/[R]_t$ and $[S]_t/([R]_t + [S]_t)$ were common to all data acquired with native membranes on the one hand and alkylated membranes on the other; the fitted estimates are listed in the table. Separate values of $[R]_t + [S]_t$ were assigned to the data from each experiment and, in most cases, to data acquired under different conditions within the same experiment. Individual estimates then were averaged to obtain the means (±SEM) listed in the table (native membranes, $N = 17$; alkylated membranes, $N = 6$). Single values of NS were common to data from the same experiment, and the mean is 0.0092 ± 0.0003. [b] The units of $[R]_t$, $[S]_t$, and $[G_j]_t$ as well as those of related parameters in Table 2 (*i.e.*, K_{Gj} and $K_{Gj.N}$) are with respect to the total volume of the homogenate. They cannot be taken literally, since receptors and G proteins are localized within the membrane. [c] The means for selected experiments as grouped in Figure 2 are as follows: (A and D) ($N = 6$), 61 ± 3 pM (no GMP-PNP), 61 ± 3 pM (0.1 mM GMP-PNP); (C) ($N = 5$), 83 ± 7 pM; (B) ($N = 3$), 11 ± 1 pM (no GMP-PNP), 12 ± 1 pM (0.1 mM GMP-PNP).

to only 2.4 after alkylation, a 6.2-fold decrease in $[R]_t$ is accompanied by 2.7-fold decrease in $[G_1]_t$ and $[G_2]_t$ (Table 3). Scheme 1 describes a spontaneously reversible association between R and G_j, and the density of G proteins is expected to be independent of the mustard. Alkylation therefore is expected to increase the value of $([G_1]_t + [G_2]_t)/[R]_t$ to 6.5. Forced equivalence between the relative values of $[R]_t$ and $([G_1]_t + [G_2]_t)/[R]_t$ leads to a large increase in the sum of squares ($P < 0.00001$) and to marked deviations between the fitted curves and the data.

The anomalous decrease in $[G_j]_t$ derives from the refractory nature of the binding patterns. In the absence of nucleotide, carbachol increased the affinity of R for G by about 630-fold in the case of G_1 and by more than 10000-fold in the case of G_2 ($K_A/K_{A.Gj} = K_{Gj}/K_{Gj.A}$) (Table 2). Native membranes contained equimolar amounts of R and total G, and the free concentrations of the latter were decreased at least 13-fold upon coupling.[2] In the alkylated membranes, however, G proteins are expected to outnumber those receptors untouched by the mustard (*i.e.*, $[G_1]_t/[R]_t = 5.0$, $[G_2]_t/[R]_t = 1.5$). The potential for agonist-induced depletion therefore is less, and the unreacted receptors ought to experience less competition when forming the RG complex. Such an effect will emerge as an increase in the apparent affinity of carbachol, and the binding patterns predicted for the alkylated membranes are shown by the dashed lines in Figure 2B. The failure of a decrease in $[R]_t$ to effect the leftward shift required by Scheme 1 emerges as a decrease in $[G_j]_t$ under the conditions of the analysis. In fact, the inhibitory potency of carbachol was largely unchanged by the mustard. In terms of the multisite model, alkylation had little or no effect on apparent affinity in the absence of nucleotide (K_{Aj}) and affected only K_{A2} in the presence of GMP-PNP [Table 2 in Chidiac *et al.* (1997)].

Receptors tagged irreversibly with propylbenzilylcholine were not considered explicitly in the analysis illustrated in Figure 2. According to Scheme 1 and the parametric values listed in Tables 2 and 3, about 39% of the receptors identified as R were coupled to G_1 in preparations of native membranes

[2] The magnitude of the effect can be calculated from the parametric values listed in Tables 2 and 3. At native levels of receptor (*i.e.*, $[R]_t = 60$ pM, $[S]_t = 7.9$ pM, Figure 2A), the free concentration of G_1 in the presence of 1.0 nM *N*-[^{3}H]methylscopolamine is decreased from 43 pM in the absence of agonist to 3.3 pM in the presence of 10 mM carbachol. The corresponding values for G_2 are 14 and 0.12 pM, respectively. At reduced levels of receptor under otherwise identical conditions (*i.e.*, $[R]_t = 9.6$ pM, $[S]_t = 2.2$ pM), the free concentration of G_1 is decreased only slightly, from 47 to 44 pM, and that of G_2 is decreased from 15 to 8.5 pM.

devoid of ligands; similarly, 8.5% were coupled to G_2. It therefore is implicit in the analysis that the covalently bound antagonist promotes dissociation of the complex, at least in the absence of guanyl nucleotide. If the mustard were without effect on coupling, the discrepancy between observed and predicted behavior might be less than that illustrated in Figure 2B. In the presence of GMP-PNP, however, >86% of the receptors were uncoupled; 13.5% occurred as RG_2N, and the levels of RG_1, RG_2, and RG_1N were negligible (<0.02%). Since the discrepancy occurs with and without the nucleotide, a rationalization in terms of Scheme 1 would require that alkylation increase the affinity of the receptor for the G protein.

Effects of Carbachol and Guanyl Nucleotides on the Binding of N-[^{3}H]Methylscopolamine at High Ionic Strength. Data acquired in buffer B were combined and analyzed in the manner described above for buffer A. The correlation between $[G_j]_t/[R]_t$ and $[S]_t/([R]_t + [S]_t)$ is stronger than at lower ionic strength, and the value of $[S]_t/([R]_t + [S]_t)$ therefore was fixed at 0.118 throughout (see Table 3). The affinity of R for G_j or G_jN is poorly defined in all cases. Maps of K_{Gj} and $K_{Gj.N}$ reveal only a lower bound and indicate that there was little or no coupling of receptor and G protein in the absence of agonist, as expected from the hyperbolic binding of *N*-[^{3}H]methylscopolamine and its insensitivity to GMP-PNP in buffer B (Chidiac *et al.*, 1997). The values of K_{Gj} and $K_{Gj.N}$ therefore were fixed arbitrarily at 0.1 μM when required to stabilize the fit. Included in the analysis were data on the dose-dependent effects of three nucleotides: GMP-PNP, GTPγS, and GDP. The corresponding estimates of K_{Nj} are well-defined and largely independent of the affinity of carbachol for the receptor or of the receptor for the G protein. The data and fitted curves are illustrated in Figure 3, and the corresponding parametric values are listed in Tables 4 and 5.

Agreement between the model and the inhibitory effect of carbachol is better than that obtained at low ionic strength (Figure 3A). The improvement is due largely to the relative scarcity of high-affinity sites when GMP-PNP is saturating. Binding at graded concentrations of GMP-PNP or GTPγS is well described by the model (Figure 3B), which interprets the two inflections as a difference in the affinity of the nucleotide for G_1 and G_2 (K_{Nj}, Table 4). Scheme 1 also can describe the downward inflection observed at about 0.6 μM GDP, which emerges as a nucleotide-promoted increase in the affinity of the receptor for G_2 (*cf.* K_{G2} and $K_{G2.N}$, Table 4) and in the affinity of carbachol for the receptor (*cf.* $K_{A.G2}$ and $K_{A.G2N}$). In contrast, the model cannot follow the upward inflection occurring at about 0.3 mM GDP and characterized by a Hill coefficient of 1.4 (Chidiac *et al.*, 1997). Since all G proteins compete for a single site on the receptor, Scheme 1 cannot account for a Hill coefficient >1 regardless of the degree of heterogeneity represented by G_j.

Although otherwise consistent with the expected behavior of the system, the analysis summarized in Figure 3 placed no constraint on the relative numbers of G_1 and G_2 recognized by different guanyl nucleotides. The fitted estimates of $[G_j]_t/[R]_t$ are listed in Table 5, and no two nucleotides yield the same values of $[G_1]_t$ and $[G_2]_t$; conversely, single values of $[G_j]_t/[R]_t$ increase the global sum of squares by 17% ($P < 0.00001$), and the fitted curves fail to provide even a first approximation of the data. Since GMP-PNP, GTPγS, and GDP all were tested in parallel, the differences cannot be attributed to variations among animals or between experiments. There is no provision in Scheme

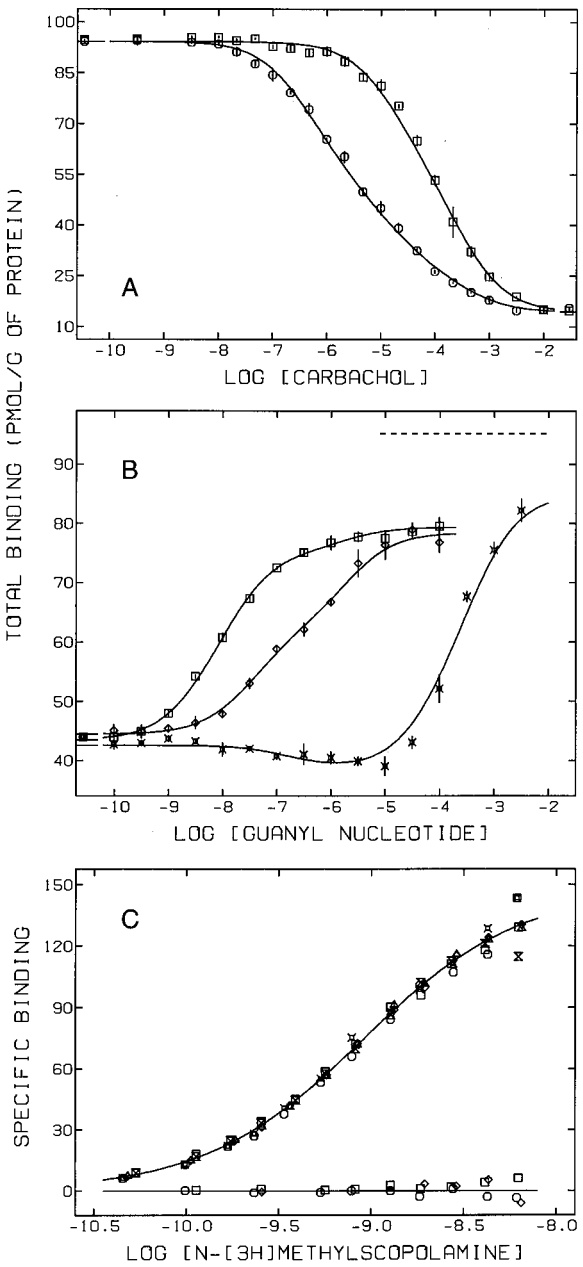


FIGURE 3: Fit of Scheme 1 to the binding of *N*-[^{3}H]methylscopolamine, carbachol, and guanyl nucleotides in preparations of native membranes at high ionic strength. Total binding was measured following equilibration of the membranes in buffer B at graded concentrations of the ligand shown on the abscissa. Each panel contains the combined data from three experiments, and the conditions were as follows: (A) 1.02−1.14 nM [^{3}H]NMS, no GMP-PNP (○), 0.1 mM GMP-PNP (□); (B) 1.01−1.09 nM [^{3}H]NMS and 10 μM carbachol plus GMP-PNP (□), GTPγS (◊), or GDP (⊗); (C) [^{3}H]NMS alone (○, ◊, □) and plus 0.1 mM GMP-PNP (⊗, △, hourglass) (upper curve), [^{3}H]NMS plus 0.01 mM unlabeled NMS (baseline). Different symbols in panel C denote data from different experiments (○, ⊗; ◊, △; □, hourglass). The dashed line in panel B indicates the binding of [^{3}H]NMS in the absence of agonist and guanyl nucleotide (95.2 ± 1.2 pmol/g of protein). The solid lines represent the best fit of the model ($n = 2$) to the data taken together, and the parametric values are listed in Tables 4 and 5. Values plotted on the ordinate were obtained according to eq 6 in Chidiac *et al.* (1997), and the mean value of $[P]_t$ used for panels A and B is 1.06 ± 0.02 nM. Points shown at the lower and upper ends of the abscissa indicate binding in the absence of unlabeled ligand (A, B) and in the presence of 0.01 mM unlabeled NMS (A), respectively.

Table 4: Affinities Inferred from the Binding of *N*-[^{3}H]Methylscopolamine at High Ionic Strength: Analysis in Terms of Scheme 1 ($n = 2$)[a]

reactants	parameter[b]	value
carbachol + R	log K_A	-3.72 ± 0.10
carbachol + RG_1	log $K_{A.G1}$	-9.87 ± 0.04
carbachol + RG_2	log $K_{A.G2}$	-8.60 ± 0.53
carbachol + RG_1·GDP	log $K_{A.G1N}$	*c*
carbachol + RG_2·GDP	log $K_{A.G2N}$	-9.57 ± 1.05
[^{3}H]NMS + R	log K_P	-9.07 ± 0.01
GMP-PNP + G_1	log K_{N1}	-9.23 ± 0.08
GMP-PNP + G_2	log K_{N2}	-5.82 ± 0.57
GTPγS + G_1	log K_{N1}	-8.47 ± 0.15
GTPγS + G_2	log K_{N2}	-5.90 ± 0.27
GDP + G_1	log K_{N1}	-4.84 ± 0.07
GDP + G_2	log K_{N2}	-5.71 ± 0.50
receptor + G_1	log K_{G1}	-7.0[b]
receptor + G_2	log K_{G2}	-6.85 ± 0.53
receptor + G_1·GDP	log $K_{G1.N}$	-7.0[b]
receptor + G_2·GDP	log $K_{G2.N}$	-7.66 ± 1.01

[a] Equation 1 was fitted to estimates of total binding in preparations of native membranes. The analysis involved 25 sets of data in which [^{3}H]NMS, carbachol, or a guanyl nucleotide (GMP-PNP, GTPγS, or GDP) was the variable ligand. The data were from nine experiments performed as follows, with the number of repeats shown in parentheses: graded concentrations of the radioligand alone, plus 0.1 mM GMP-PNP, and plus 0.01 mM unlabeled NMS (3) (Figure 3C); 1.02−1.14 nM [^{3}H]NMS and graded concentrations of carbachol, alone and together with 0.1 mM GMP-PNP (3) (Figure 3A); 1.01−1.09 nM [^{3}H]NMS plus 10 μM carbachol and graded concentrations of GMP-PNP, GTPγS, and GDP (3) (Figure 3B). One of the experiments at graded concentrations of carbachol also included parallel samples containing 1.0 mM GDP. The experiments at graded concentrations of guanyl nucleotide included estimates of nonspecific binding and of binding in the absence of both carbachol and nucleotide. Except as described in the text, parameters were assigned to force internal consistency in terms of the model. Single values of K_P and K_{Gj} were common to all of the data. Single values of K_A and $K_{A.Gj}$ also were common to all data, except as noted below; similarly, single values of $K_{A.GjN}$ were common to all data acquired without nucleotide and with GMP-PNP, on the one hand, and to data acquired with GDP on the other. The parameters K_A, $K_{A.Gj}$, and $K_{A.GjN}$ are not relevant to binding at graded concentrations of [^{3}H]NMS. Single values of $K_{Gj.N}$ and K_{Nj} were common to all data acquired without nucleotide and with GMP-PNP, to data acquired with GTPγS, or to data acquired with GDP. The estimates of capacity are listed in Table 5. The analysis was constrained to preclude solutions that predict nucleotide-dependent effects on the binding of [^{3}H]NMS. [b] There is a negative correlation between K_{Gj} and $K_{A.Gj}$, and between $K_{Gj.N}$ and $K_{A.GjN}$. Also, K_{Gj} and $K_{Gj.N}$ are defined only by lower bounds in most cases; the values therefore were fixed at -7.0 for GMP-PNP and as shown. The fitted values of $K_{A.G1N}$ and $K_{A.G2N}$ in the presence of GMP-PNP are -6.65 ± 0.90 and -8.24 ± 0.13, respectively. The binding of carbachol was not measured at a saturating concentration of GTPγS, and the corresponding values of $K_{A.GjN}$ were taken as defined by GMP-PNP. The fitted values of log $K_{G1.N}$ and log $K_{G2.N}$ for GTPγS are -6.95 ± 0.91 and -6.65 ± 0.09, respectively. [c] The value is undefined by the data.

Table 5: Capacities Inferred from the Binding of *N*-[^{3}H]Methylscopolamine at High Ionic Strength: Analysis in Terms of Scheme 1 ($n = 2$)[a]

	nucleotide		
parameter	GMP-PNP	GTPγS	GDP
$[G_1]_t/[R]_t$	0.69 ± 0.03	0.51 ± 0.05	0.85 ± 0.02
$[G_2]_t/[R]_t$	0.64 ± 0.17	1.3 ± 0.4	0.11 ± 0.02
$([G_1]_t + [G_2]_t)/[R]_t$	1.3	1.8	0.94
$[G_1]_t$ (pM)	45	33	54
$[G_2]_t$ (pM)	42	87	7.3
$[G_1]_t + [G_2]_t$ (pM)	87	120	61

[a] Equation 1 was fitted to estimates of total binding in preparations of native membranes, as described in the footnotes to Table 4. Capacities were estimated in terms of the parameters $[R]_t + [S]_t$, $[S]_t/([R]_t + [S]_t)$, and $[G_j]_t/[R]_t$. Single values of $[R]_t + [S]_t$ were common to all data acquired in the same experiment, and the mean from the nine experiments is 74 ± 2 pM. The value of $[S]_t/([R]_t + [S]_t)$ was fixed at 0.118 as described in the text. Single values of $[G_1]_t/[R]_t$ and $[G_2]_t/[R]_t$ were common to all data acquired without nucleotide and with GMP-PNP, to data acquired with GTPγS, or to data acquired with GDP to obtain the fitted estimates listed in the table. Single values of NS were common to data from the same experiment, and the mean is 0.0068 ± 0.0004.

1 for ligand-dependent effects on capacity. The system therefore cannot be described by the model with only two classes of G protein.

Effects of Guanyl Nucleotides and Carbachol on the Binding of [^{35}S]GTPγS at High Ionic Strength. Binding of [^{35}S]GTPγS to myocardial membranes has been reported to reveal three classes of sites, at least two of which appear to be G proteins linked to muscarinic receptors (Chidiac and Wells, 1992). The inhibitory behavior of GMP-PNP, GDP, and unlabeled GTPγS at about 170 pM [^{35}S]GTPγS is illustrated in Figure 4. Each nucleotide recognized three classes of sites in terms of the multisite model [*i.e.*, Scheme 1 in Chidiac *et al.* (1997)] ($P \leq 0.0004$), and the fraction of specific binding attributable to each class differs significantly from ligand to ligand ($P < 0.0001$). A saturating concentration of carbachol was without effect on the binding of GMP-PNP or GTPγS (Figure 4A,B) but promoted an apparent redistribution of labeled sites from high to intermediate affinity for GDP (Figure 4C). Agonist-sensitive binding of GDP has been reported previously (Tota *et al.*, 1987; Hilf *et al.*, 1989; Chidiac and Wells, 1992), and the effect is blocked by atropine. There was no effect of isoproterenol at a concentration of 10 μM. The implication that guanyl nucleotides and carbachol promote an interconversion of G proteins among multiple states is inconsistent with the notion of independent sites.

Best fits of Scheme 1 are illustrated by the lines in Figure 4, and the parametric values are listed in Table 6. The effect of carbachol on the binding of GDP suggests that the sites of high and intermediate affinity are G proteins accessible to muscarinic receptors. There was no change in the apparent number of low-affinity sites (*e.g.*, F'_3 in Table 6), which therefore appear not to interact with the receptors. It was assumed that a common phenomenon underlies the multiple states revealed by all nucleotides, notwithstanding the insensitivity of GMP-PNP and GTPγS to carbachol, and low-affinity binding was interpreted throughout as a separate population of receptor-inaccessible sites (*i.e.*, S in Scheme 1). The work of previous investigators (Gilman, 1987) and the results summarized in Tables 1, 2, and 4 suggest that guanyl nucleotides promote dissociation of the RG complex. Sites of high and intermediate affinity thus correspond to free G protein and the RG complex, respectively, when the radioligand is [^{35}S]GTPγS. No nucleotide shows appreciable improvement in the goodness of fit with two classes of G protein rather than one. The pattern thus differs from that observed in the binding of *N*-[^{3}H]methylscopolamine, where at least two classes are required to describe the effects of any nucleotide tested (Figures 2C and 3B).

Scheme 1 can describe the inhibitory effect of any one nucleotide, as illustrated by the fitted curves in Figure 4.

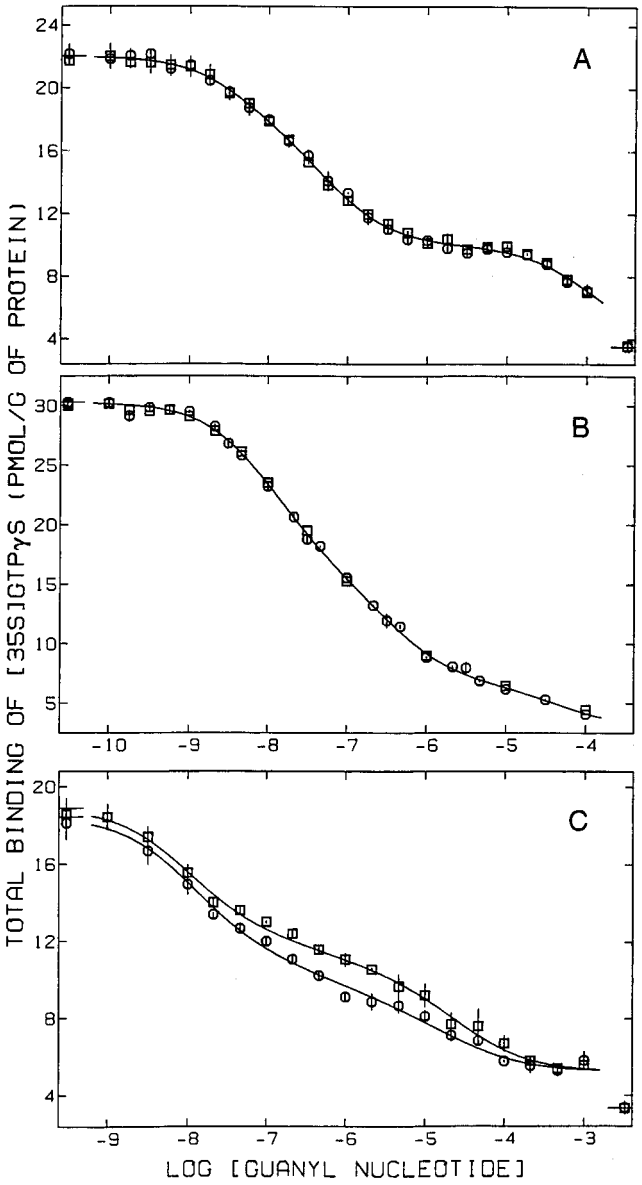


FIGURE 4: Fit of Scheme 1 to the binding of [^{35}S]GTPγS, unlabeled guanyl nucleotides, and carbachol in preparations of native membranes at high ionic strength. Total binding was measured following equilibration of the membranes (buffer B) and [^{35}S]-GTPγS (160−185 pM) with GMP-PNP (A), unlabeled GTPγS (B), or GDP (C) in the absence of agonist (○) and in the presence of 2.0 mM carbachol (□). The lines represent the best fit of the model ($n = 1$) to the data, and the parametric values are listed in Table 6. Values plotted on the ordinate were obtained according to eq 6 in Chidiac *et al.* (1997), and the mean values of [[^{35}S]GTPγS] are 170 pM (A, B) and 171 pM (C). Points shown at the lower and upper ends of the abscissa indicate binding in the absence of unlabeled ligand and in the presence of 0.1 mM GTPγS.

The effect of carbachol on the binding of GDP emerges primarily as an increase in the affinity of R for G (*cf.* K_G and $K_{G.A}$ in Table 6), in agreement with the notion that agonists favor RG over R. Also, the value of $[R]_t/[G]_t$ is 1 with each nucleotide tested. In some cases, however, nucleotide-related differences are found where none is expected. The parameters K_G and $K_{G.A}$ both represent interactions involving unliganded G proteins, but the difference found with GDP does not occur with GTPγS. Similarly, the value of K_G is larger with GTPγS than with GMP-PNP or GDP. Guanyl nucleotides also affect the inferred capacity for [^{35}S]GTPγS, as indicated by the values of $[G]_t$ and $[S]_t$ listed in Table 6. Absolute levels of specific binding varied comparatively little among the several experiments, and such variations that occurred were inconsistent with the differences in capacity;[3] rather, the differences lie primarily in the inferred distribution of sites between the two classes (*i.e.*, $[S]_t/([G]_t + [S]_t)$. As evident in the values of F'_3 (Table 6), GMP-PNP recognized proportionately more of the labeled sites as S than did either GTPγS or GDP.

DISCUSSION

Properties and Implications of Scheme 1. The notion of a ligand-regulated association between receptor and G protein is an attractive starting point for mechanistic forays into G protein-mediated signaling. Even the most frugal schemes predict the intriguing and much studied properties that are characteristic of such systems: namely, multiple states of affinity and the allosteric effects between agonists and guanyl nucleotides. Both phenomena arise from the differential affinity of the ligand for free receptor or free G protein on the one hand and the RG complex on the other. Binding to either protein creates a closed loop comprising free and coupled states, and the system is expected to behave in accord with the principle of microscopic reversibility: compounds with higher affinity for free R or G must promote uncoupling, and those with higher affinity for the complex must promote coupling. Since the system is symmetrical, comparatively few parameters are required to attempt a complete description of the interactions among receptors, G proteins, and their respective ligands.

Scheme 1 is an extension of the ternary complex model proposed originally by De Lean *et al.* (1980). The guanyl nucleotide appears as an explicit variable in addition to the agonist and radiolabeled antagonist, as in an earlier paper by Ehlert and Rathbun (1990), and the pool of receptor-accessible G proteins is potentially heterogeneous. Also included is a subpopulation of receptors inaccessible to G proteins. These extensions may be superfluous with the data from single experiments, but they are required to achieve internal consistency when the system is examined from different points of view. The extended model provides at least a first approximation of the binding properties of native membranes, and it avoids in particular the nucleotide-dependent effects on capacity that are characteristic of earlier proposals. It nevertheless fails to provide a mechanistically consistent description of the system, and the problem may lie in the presumed nature of the association between receptor and G protein.

Cardiac muscarinic receptors are commonly reported to reveal three classes of sites for agonists [*e.g.*, Uchida *et al.* (1984), Mattera *et al.* (1985), Wong *et al.* (1986), Burgen (1987), Chidiac *et al.* (1991), and Vogel *et al.* (1995)]. In terms of the mobile receptor model, three classes of sites could denote a heterogeneous population of G proteins or a

[3] The level of binding varied somewhat from batch to batch of membranes. For each experiment, the specific binding of [^{35}S]GTPγS in the absence of unlabeled guanyl nucleotide can be estimated from the fitted asymptotic values of eq 1 and adjusted to account for small differences in the free concentration of the radioligand. The means (±SEM) relative to that from the experiments with GMP-PNP are as follows: 1.00 ± 0.02 (GMP-PNP), 1.50 ± 0.36 (GTPγS), and 0.81 ± 0.07 (GDP). In contrast, the relative values of $[R]_t + [S]_t$ are as follows: 1.00 ± 0.03 (GMP-PNP), 0.57 ± 0.17 (GTPγS), and 0.30 ± 0.05 (GDP).

Table 6: Affinities and Capacities Inferred from the Binding of [^{35}S]GTPγS at High Ionic Strength: Analysis in Terms of Scheme 1 ($n = 1$)[a]

unlabeled nucleotide	carbachol (mM)	log K_N	log $K_{N.R}$ or log $K_{N.AR}$[b]	log K_G or log $K_{G.A}$[b]	log $K_{G.N}$ or log $K_{G.AN}$[b,c]	log K_{NS}	$[G]_t$ (nM)	$[S]_t$ (nM)	$[R]_t$ (nM)	F'_3[d]
GMP-PNP	0.0	-8.37 ± 0.19	-7.25 ± 0.12	[e]	[e]	-3.92 ± 0.04	10	920	10	0.35
GMP-PNP	2.0									
GTPγS	0.0	-8.14 ± 0.04	-6.33 ± 0.08	-11.15 ± 0.12	-9.33	-4.33 ± 0.18	11	510	110.14	
GTPγS	2.0									
GDP	0.0	-8.13 ± 0.12	[f]	-11.54 ± 0.08	[f]	[g]	7.1	268	7.0	0.12
GDP	2.0		-4.19 ± 0.38[f]	-11.91 ± 0.21	-7.97[f]	[g]	9.0	266	8.9	0.12

[a] Equation 1 was fitted to estimates of total binding in preparations of native membranes incubated with 160−185 pM [^{35}S]GTPγS and graded concentrations of unlabeled nucleotide. Experiments with GMP-PNP and GDP were performed in triplicate, with parallel assays in the absence of agonist and in the presence of 2.0 mM carbachol. The binding of unlabeled GTPγS was measured three times in the absence of agonist and once in parallel assays with and without carbachol. Parametric values listed in the table are from three analyses, one for each nucleotide. Labeled and unlabeled GTPγS were assumed to be functionally identical, and parameters representing affinity were constrained accordingly in the case of isotopic dilution (*i.e.*, $K_N = K_P$, $K_{N.R} = K_{P.R}$, $K_{NS} = K_{PS}$); the fitted values then were substituted for the affinity of the probe and taken as constants in other analyses. GMP-PNP and GTPγS were insensitive to carbachol, and the relevant parameters were constrained accordingly (*i.e.*, $K_{N.R} = K_{N.AR}$, $K_G = K_{G.A}$). Capacities were estimated in terms of the parameters $[G]_t + [S]_t$, $[R]_t/[G]_t$, and $[S]_t/([G]_t + [S]_t)$. The fitted values of $[R]_t/[G]_t$ are 0.9885 ± 0.0047 (GMP-PNP), 0.9812 ± 0.0047 (GTPγS), and 0.9878 ± 0.0040 (GDP); those of $[S]_t/([G]_t+[S]_t)$ are 0.9888 ± 0.0017 (GMP-PNP), 0.9789 ± 0.0085 (GTPγS), and 0.9742 ± 0.0041 or 0.9672 ± 0.0045 (GDP ∓ carbachol). Single values of $[G]_t + [S]_t$ and NS were common to all data acquired within the same experiment, and the means are as follows: 928 ± 24 nM and 0.010 ± 0.002 (GMP-PNP), 526 ± 155 nM and 0.0086 ± 0.0009 (GTPγS), 275 ± 42 nM and 0.0087 ± 0.0005 (GDP). The mean capacity and the corresponding estimates of $[S]_t/([G]_t + [S]_t)$ and $[R]_t/[G]_t$ were used to obtain the values of $[G]_t$, $[S]_t$, and $[R]_t$ listed in the table. The data and fitted curves are illustrated in Figure 4. [b] The parameters are defined as follows, where A represents carbachol: $K_{N.R} = [RG][N]/[RGN]$, $K_{N.AR} = [ARG][N]/[ARGN]$, $K_G = [R][G]/[RG]$, $K_{G.A} = [AR][G]/[ARG]$, $K_{G.N} = [R][GN]/[RGN]$, $K_{G.AN} = [AR][GN]/[ARGN]$. [c] Calculated as follows: $\log K_{G.N} = \log K_G - \log K_N + \log K_{N.R}$, $\log K_{G.AN} = \log K_{G.A} - \log K_N + \log K_{N.AR}$. [d] The fraction of observed specific binding attributed to sites of type S. An expression of the form $\sum_{j=1}^{3} F_j' K_j^{n_{H(j)}}/(K_j^{n_{H(j)}} + [A]^{n_{H(j)}})$ (*cf.* eq 1 in Chidiac *et al.*, 1997) was fitted to simulated data corresponding to the fitted curves in Figure 4. [e] The values of $\log K_G$ and $\log K_{G.N}$ are highly correlated and defined only by upper bounds (*i.e.*, $\log K_G < -11.4$, $\log K_{G.N} < -10.0$), as indicated by the sum of squares ($P = 0.05$) (*cf.* Figure 1). Other parameters were estimated with $\log K_G$ taken as −13.5 ($\log K_{G.N} = -12.37$), and the values are independent of K_G at that point (*i.e.*, $\log K_G \ll -11.4$). [f] The values of $\log K_{N.R}$ and $\log K_{G.N}$ are highly correlated and defined only by lower bounds (*i.e.*, $\log K_{N.R} > -4.6$, $\log K_{G.N} > -8.3$) ($P = 0.05$), as indicated by the sum of squares; the minimum for $K_{N.AR}$ is shallow toward higher values of the parameter. Other parameters were estimated with $\log K_{N.R}$ taken as −2 ($\log K_{G.N} = -5.40$), and the values are independent of $K_{N.R}$ at that point. The affinity of GDP for the RG complex is unaffected or increased by carbachol at values of $K_{N.R}$ that yield the best fit (*i.e.*, $K_{N.R} \geq K_{N.AR}$). [g] The value is not defined by the data.

restriction on the fraction of receptors that can form an RG complex. The latter possibility could arise from an excess of receptors over G proteins within the relevant pool or, as required here, from a subpopulation of receptors that cannot interact with G proteins (Lee *et al.*, 1986; Vogel *et al.*, 1995). Either way, the G protein-free receptors account for the sites of lowest affinity, and the fitted value of K_{A3} from the multisite model equals or closely approximates the value of K_A from Scheme 1; receptors accessible to G proteins account for the sites of highest and intermediate affinity, and one or both of K_{A1} and K_{A2} are expected to differ from $K_{A.G}$ and K_A (*i.e.*, $K_{A.G} < K_{A1} < K_{A2} < K_A$; Lee *et al.*, 1986).

In previous attempts to apply the mobile receptor model to cardiac muscarinic receptors, the sites of lowest affinity were attributed by default to receptors that outnumber G proteins (Ehlert, 1985; Wong *et al.*, 1986; Ehlert and Rathbun, 1990; Minton and Sokolovsky, 1990). In one study, two classes of competing G proteins were required to account for the effects of GMP-PNP and batrachotoxin (Minton and Sokolovsky, 1990). The model also has been used to describe the kinetics of the interaction between α_2-adrenergic receptors and the agonist UK 14,304, which was found to recognize two classes of sites in human platelets (Neubig *et al.*, 1985, 1988). Total G was assumed to exceed total R within the relevant pool, which accounted for the sites of high affinity, and the sites of low affinity were taken as receptors inaccessible to G proteins (Neubig *et al.*, 1988). A_1 adenosine receptors have been reported to reveal three classes of sites recognized by agonists and antagonists, which displayed an opposite preference similar to that shown for carbachol and *N*-[^{3}H]methylscopolamine [Leung *et al.*, 1990; *cf.* Table 1 in Chidiac *et al.* (1997)]. The data could be described equally well by assuming that two G proteins compete for one receptor or that two receptors compete for one G protein (Leung *et al.*, 1990).

Cardiac muscarinic receptors are predominantly if not exclusively M_2 [see references in Chidiac *et al.* (1997)], and only one class of receptor is accessible to G proteins in Scheme 1. With only one class of G protein, however, the model could not provide even a first approximation of the present data. The need for greater complexity arises in part from the dose-dependent effects of guanyl nucleotides on the binding of carbachol (Figures 2C and 3B). A single class cannot account for the dispersions revealed by GTPγS and GMP-PNP, which are too broad to derive wholly from changes in the mutual depletion of R and G, or for the U-shaped effect of GDP. One class of G protein also cannot account simultaneously for the binding of carbachol to native and alkylated membranes, even allowing for changes in total G. The discrepancies reflect the failure of propylbenzilylcholine mustard to affect the value of K_{A1} in the multisite model, and they occur regardless of whether or not all of the receptors can form an RG complex. The sites designated G_1 and G_2 might derive from α_i and α_o, which have been identified in western blots of immunoprecipitated and purified cardiac muscarinic receptors (Matesic *et al.*, 1991; Wreggett and Wells, 1995).

The requirement for G protein-free receptors reflects the degree of heterogeneity recognized by agonists, as described above, and it arises from the failure of propylbenzilylcholine mustard to eliminate the sites corresponding to those of lowest affinity in terms of the multisite model. If the ratio

of G proteins to receptors is indeed near 1 in native membranes (Tables 1 and 3), and if all receptors are accessible to G proteins, there ought to be at least 5 G proteins per functional receptor following a decrease of at least 80% in the density of the latter. As noted previously [*e.g.*, Lee *et al.* (1986)], the Hill coefficient is expected to be indistinguishable from 1 whenever G proteins outnumber receptors by about 2-fold or more.

In terms of Scheme 1, GMP-PNP is without effect on $[G_j]_t$ in native or alkylated membranes. Taking only data from native membranes (*i.e.*, Figure 2A,D), a similar result can be obtained with two classes of G proteins accessible to all receptors (*i.e.*, $[S]_t/([R]_t + [S]_t) = 0$) or with one class of G proteins accessible to a subclass of receptors (*i.e.*, $[S]_t/([R]_t + [S]_t) > 0$). With one class of G proteins accessible to all receptors, however, the effect of GMP-PNP emerges as a decrease in the number of G proteins. Such a loss implies that GMP-PNP acts irreversibly, and the same result has been reported previously for the effect of the nucleotide on the binding of agonists to β-adrenergic (De Lean *et al.*, 1980), D_2-dopaminergic (Wreggett and De Lean, 1984), and cardiac muscarinic receptors (Wong *et al.*, 1986; Minton and Sokolovsky, 1990).

The notion of an irreversible process recalls evidence that the binding of guanyl nucleotides is not necessarily reversible on the time scale of binding assays: G proteins typically are purified in a GDP-bound form (Birnbaumer *et al.*, 1990), and the dissociation of [^{35}S]GTPγS can be unmeasurably slow under some conditions [*e.g.*, Higashijima *et al.* (1987)]. Similar effects do not appear to occur with GMP-PNP, which has been shown to bind reversibly under the conditions of typical assays (Ross *et al.*, 1977; Michel and Lefkowitz, 1982; Wells and Cybulsky, 1990). Moreover, concentrations of the nucleotide that are subsaturating but nonlimiting achieve intermediate effects on the binding of agonists at what appears to be thermodynamic equilibrium [*e.g.*, De Lean *et al.* (1980) and Galper *et al.* (1987)]. Finally, the binding of both GDP and GTPγS becomes independent of time under the conditions of the present experiments, as expected for a system at equilibrium, and that of [^{35}S]GTPγS is largely reversible (Chidiac and Wells, 1992).

Monovalent cations allosterically modulate the binding properties of receptors that inhibit adenylate cyclase. The effect on α_2-adrenergic receptors has been localized to aspartate-79, a residue that is highly conserved among all G protein-coupled receptors (Horstman *et al.*, 1990). The two buffers used in the present investigation differed primarily in their concentration of sodium chloride, and that may account for observed differences in the binding properties. Binding was generally weaker in buffer B, and there were fewer high-affinity sites in the absence of GMP-PNP (Chidiac *et al.*, 1997). In terms of Scheme 1, a higher concentration of salt was associated with reduced affinity of the free receptor for *N*-[^{3}H]methylscopolamine (K_P), carbachol (K_A), and both G proteins (K_{G1}, K_{G2}) (Tables 2 and 4). The effect on the affinity of the agonist for RG_j and RG_jN is less clear, owing to uncertainty over the values of K_{Gj} and $K_{Gj.N}$ at higher ionic strength. The affinity of GMP-PNP for the free G protein is increased in one case and decreased in the other. All of the foregoing are consistent with Scheme 1, although sodium chloride is not included specifically as a variable.

Anomalous Behavior Identified through Labeling of the Receptor. The present data call into question the basic notion of a ligand-regulated equilibrium between free and G protein-coupled receptors. At low ionic strength, neither GMP-PNP nor propylbenzilylcholine mustard had an appreciable effect on the value of K_{A1} obtained for carbachol in terms of the multisite model (Chidiac *et al.*, 1997). The data therefore are at variance with Scheme 1, in which the nucleotide affects the affinity of the receptor for the G protein and the mustard reduces the concentration of functional receptors. Either effect is expected to perturb the equilibrium between coupled and uncoupled receptors, with attendant shifts in the inhibitory potency of agonists (Lee *et al.*, 1986). Those shifts do not occur, and the model is unable to describe the high-affinity sites recognized by agonists in native membranes at saturating concentrations of GMP-PNP (Figure 2A); it cannot account for the effects of alkylation irrespective of GMP-PNP (Figure 2B). The first discrepancy has been noted previously (Wong *et al.*, 1986; Ehlert and Rathbun, 1990), and it persists here despite the avoidance of an untoward loss of G proteins in the presence of the nucleotide.

The inability of Scheme 1 to account for the effects of alkylation emerges as a decrease in the inferred densities of G_1 and G_2 (Table 3). A similar anomaly is seen in the different values of total G_j obtained with different guanyl nucleotides (Table 5). It is implicit in Scheme 1 that the RG complex dissociates rapidly and spontaneously on the time scale of a binding assay, and the numbers of G proteins ought to be independent of the numbers of receptors. Also, G_1 and G_2 are defined as noninterconverting, and the number of each ought to be independent of the nucleotide. While these anomalies signal the inadequacy of the model, they are determined arbitrarily by the constraints applied during the fitting procedure. The densities of G_1 and G_2 are not estimated directly when *N*-[^{3}H]methylscopolamine is the probe; accordingly, discrepancies with the data tend to emerge in those parameters when all others are assigned to enforce mechanistic consistency. Deviations between the fitted curves and the data occur to the extent that inconsistencies cannot be absorbed by capacity alone. This is particularly evident in the failure of Scheme 1 to account for high-affinity binding in the presence of GMP-PNP, as described above, or for the Hill coefficient of 1.4 revealed by GDP.

It is assumed in the analyses with Scheme 1 that an irreversibly bound antagonist precludes the interaction between receptor and G protein. The question may be irrelevant in the presence of GMP-PNP, which itself promotes dissociation of the complex, but the model suggests that about 50% of the receptors in native membranes were coupled in the absence of nucleotide. Various observations are consistent with the notion that muscarinic agonists and antagonists hold the opposite preference for two spontaneously interconverting states of the receptor. Both *N*-methylscopolamine and atropine are inverse agonists at M_2 receptors regulating adenylate cyclase in CHO cells and rat cardiomyocytes (Jakubik *et al.*, 1995). Also, GMP-PNP increased the overall affinity for *N*-[^{3}H]methylscopolamine in the present investigation (Figure 2D), and similar effects have been reported previously for *N*-methylscopolamine (Hulme *et al.*, 1981; Potter *et al.*, 1991) and for quinuclidinylbenzilate (Burgisser *et al.*, 1982; Mattera *et al.*, 1985; Boyer *et al.*, 1986). In the context of Scheme 1, a leftward

shift in the binding curve implies that the antagonist favors the free receptor over the RG complex. Propylbenzilylcholine mustard labels a conserved aspartate that probably interacts with the onium headgroup of muscarinic antagonists (Curtis *et al.*, 1989), and it also may behave like other antagonists in its effect on the supposed equilibrium between R and RG.

As interpreted above, the apparent loss of G proteins is an artifact arising from the inadequacies of the model and the assignment of parameters during the fitting procedure. A real loss cannot be ruled out, but it seems unlikely that the G proteins themselves were alkylated by propylbenzilylcholine mustard. The reactive aziridinium ion binds specifically to muscarinic receptors, and nonspecific alkylation is expected to be low at the concentration of reagent used to prepare the treated membranes (Berrie *et al.*, 1984a). Also, the decrease in $[G_1]_t + [G_2]_t$ is equal in magnitude to almost 60% of the sites labeled by *N*-[^{3}H]-methylscopolamine in native membranes. In contrast, labeled receptors appear to have been the single major product when cortical membranes were pretreated with tritiated mustard and examined in hydrodynamic studies (Birdsall *et al.*, 1979; Hulme *et al.*, 1983; Berrie *et al.*, 1984a).

Some of the parametric values obtained with Scheme 1 seem to conflict with the results of biochemical studies. GTP and analogues such as GMP-PNP generally are believed to promote dissociation of the RG complex (Gilman, 1987; Birnbaumer *et al.*, 1990), presumably via an increase in the value of K_G. The expected change is observed with GMP-PNP and G_1, as indicated by the difference of at least 10-fold between K_{G1} and $K_{G1.N}$. In contrast, there is little or no difference between K_{G2} and $K_{G2.N}$. Also, there is a 29-fold difference in the affinity of the nucleotide for G_1 and G_2 (*i.e.*, K_{Nj} in Table 2). The divergent behavior of the two G proteins is unexpected if those of Scheme 1 correspond to G_o and G_i. Studies on purified material suggest that G_o and the different G_i's are similar, at least at equilibrium, in their interaction either with guanyl nucleotides or with muscarinic receptors (Ikegaya *et al.*, 1990; Carty *et al.*, 1990).

Labeling of the G Protein and the Lack of Symmetry. [^{35}S]-GTPγS has been shown to label G proteins linked to cardiac muscarinic receptors in native membranes (Hilf *et al.*, 1989; Chidiac and Wells, 1992) and in reconstituted preparations (Tota *et al.*, 1987). As illustrated in Figure 4C, the inhibitory behavior of GDP reveals multiple classes of sites in a pattern reminiscent of that revealed by agonists at muscarinic receptors. Moreover, carbachol effects an apparent interconversion of sites from higher to lower affinity in a manner that mimics the effect of guanyl nucleotides on the binding of agonists. The magnitude of the interconversion implies that most if not all of the labeled sites are linked to muscarinic receptors, suggesting a degree of specificity in the binding of the radioligand (Chidiac and Wells, 1992). The mutual interactions between carbachol and GDP resemble the reciprocal effects predicted by Scheme 1 when the RG complex is favored by agonists (*i.e.*, $K_{A.Gj} < K_A$) and disfavored by guanyl nucleotides (*i.e.*, $K_{Nj} < K_{Nj.R}$). Carbachol has no effect on GMP-PNP or GTPγS, however, and that on GDP is too small.

Asymmetry emerges from Scheme 1 as parametric values that ought to be the same regardless of whether the system is viewed through radioligands to the receptor or to the G protein. Although the data summarized in Tables 4 and 6 were acquired under the same conditions, apart from the radioligand, estimates of affinity differ to a degree that is wholly inconsistent with the model. Estimates of K_{Gj} obtained from assays with *N*-[^{3}H]methylscopolamine imply that there is no appreciable coupling of R and G_j in the absence of ligands to either protein (*i.e.*, $K_{Gj} \gg [R]_t \equiv [G]_t$, Table 4); in contrast, those obtained from assays with [^{35}S]-GTPγS imply that R and G are more than 95% coupled (*i.e.*, $K_{Gj} \ll [R]_t \equiv [G]_t$, Table 6). The resting state of the system thus shows an anomalous dependence upon the measurement itself. Asymmetry also is found in the effect of carbachol on the inhibition of [^{35}S]GTPγS by GDP (Figure 4C), which emerges from Scheme 1 as an increase in the affinity of the receptor for the G protein (*i.e.*, K_G and $K_{G.A}$ in Table 6). While qualitatively consistent with the notion that agonists promote coupling, the 2.4-fold difference between K_G and $K_{G.A}$ is orders of magnitude smaller than that inferred from the assays with *N*-[^{3}H]methylscopolamine (Table 4, $K_{Gj}/K_{Gj.A} = K_A/K_{A.Gj}$). Carbachol was without discernible effect on the binding of GMP-PNP and GTPγS (Figure 4A,B; $K_G = K_{G.A}$ in Table 6), which leads to the erroneous prediction that neither will affect the binding of the agonist.

Scheme 1 and related models predict multiple states of affinity only when the concentration of labeled sites is comparable to or exceeds that of either the G protein or the receptor, as appropriate (Lee *et al.*, 1986). The fitted value of $[R]_t/[G]_t$ is 1 in assays with [^{35}S]GTPγS, and the concentration of receptors inferred from the value of $[G]_t$ is 7−11 nM (Table 6). That is about 100 times the concentration measured directly with *N*-[^{3}H]methylscopolamine in assays performed under the same conditions (Table 5). Estimates of $[G]_t$ involve a long extrapolation from the low concentration of [^{35}S]GTPγS used in the experiments, but the attendant uncertainty cannot account for the discrepancy in $[R]_t$. Since the values of $[G_j]_t/[R]_t$ obtained from the binding of *N*-[^{3}H]methylscopolamine also are near 1 (Table 5), there is a similar discrepancy in the value of $[G]_t$.

Properties inferred from the binding of [^{35}S]GTPγS also lack internal consistency. Different guanyl nucleotides reveal different numbers of G proteins, as in the assays with *N*-[^{3}H]-methylscopolamine, and the Hill coefficient of 1.4 found for GDP suggests that it is inappropriate simply to increase the number of classes. In addition, the nucleotide appears to affect the affinity of the receptor for the unliganded G protein. A discrepancy also occurs in the affinity of GMP-PNP, GTPγS, and GDP for the uncoupled G protein. In each case, the single value estimated with [^{35}S]GTPγS (K_N) differs from both of the values estimated with *N*-[^{3}H]methylscopolamine (K_{Nj}).

Possible Extensions to Scheme 1. The basic premise of the mobile receptor model is that formation of the RG complex is both random and reversible. If so, extensions to Scheme 1 might resolve the issues described above. Native membranes may contain multiple compartments, each with its own distinct complement of receptors and G proteins. Also, it has been suggested that the receptor can exist in two states differing in affinity for the ligand on the one hand and the G protein on the other (Samama *et al.*, 1993; Weiss *et al.*, 1996). The G proteins themselves are heterotrimeric and may fragment. Activated G proteins are known to dissociate into the α subunit and a $\beta\gamma$ heterodimer, at least with purified material in solution (Gilman, 1987; Birnbaumer *et al.*, 1990; Lee *et al.*, 1992). Similarly, α subunits can

exchange or be released at the surface of membranes, at least under some conditions [*e.g.*, Lynch *et al.* (1986), Ransnas and Insel (1988b), Milligan *et al.* (1988), Yatani *et al.* (1988), and Carr *et al.* (1990)], and transduction generally is believed to involve the GTP-induced release of α from $\beta\gamma$ and from the receptor (Gilman *et al.*, 1987; Birnbaumer *et al.*, 1990; Conklin and Bourne, 1993). Such refinements or combinations thereof cannot be rejected in the absence of analyses in terms of explicit models.

Fragmentation of the G protein is of particular interest, since it might account for the one-sided interaction between carbachol and GMP-PNP. Various possibilities can be envisaged, of which two are encompassed by Scheme 1. The formulation of the model is the same if the dissociation into subunits occurs only when the G protein is not engaged with the receptor or if the $\beta\gamma$ heterodimer remains coupled to the receptor while the α subunit exchanges. Not encompassed by Scheme 1 are those possibilities in which α and $\beta\gamma$ bind to the receptor in a stepwise manner. If the receptor, the $\beta\gamma$ heterodimer, and the nucleotide all interact independently with the α subunit, there is no thermodynamic requirement for symmetry between the nucleotide and the agonist (Onaran *et al.*, 1993). Several lines of evidence suggest that either the α subunit or the $\beta\gamma$ heterodimer can bind to the receptor [*e.g.*, Im *et al.* (1988), Kelleher and Johnson (1988), and Phillips *et al.* (1992)], but the functional activity of the former is much enhanced in the presence of the latter (Fung, 1983; Katada *et al.*, 1986; Florio and Sternweis, 1989). Also, receptors appear to distinguish among different $\beta\gamma$ heterodimers (Fawzi *et al.*, 1991), perhaps via the β subunit (Kleuss *et al.*, 1992). It thus appears that the receptor interacts predominantly with the holo G protein, as described in Scheme 1.

Transient Complexes and Amplification. An attractive property of the mobile receptor model is the potential for amplification between the receptor and the G protein. Scheme 1 and other versions of the model consistently yield estimates of $[G]_t/[R]_t$ or $[R]_t/[G]_t$ that are near 1, regardless of whether or not the assignment of parameters is formally consistent with the supposed effects of guanyl nucleotides or propylbenzilylcholine mustard [Tables 3, 5, and 6; see also De Lean *et al.* (1980), Wreggett and De Lean (1984), Ehlert (1985, 1987), Minton and Sokolovsky (1990), and Leung *et al.* (1990)]. Indeed, values near 1 are mandatory if a ligand-regulated equilibrium between free and coupled receptors is to contribute to the low Hill coefficients typical of agonists in binding studies (Lee *et al.*, 1986). If the total numbers of receptors and G proteins are equal or nearly so, amplification requires that only some of the receptors become activated over the time of the measurement.

In studies on the rhodopsin-stimulated exchange of GMP-PNP, only 0.0055 mol of the nucleotide was found to bind per mole of total rhodopsin; since only 0.0011% of the rhodopsin was photolyzed, the turnover was 500 mol of nucleotide/mol of activated rhodopsin (Fung and Stryer, 1980). The photolysis of rhodopsin can be initiated and quenched within seconds; in contrast, the activation of muscarinic receptors typically is measured over minutes and at saturating concentrations of an agonist. As activation integrated over time approaches 100%, the turnover of nucleotide is expected to decrease to a value not exceeding $[G]_t/[R]_t$. In porcine atrial membranes, however, the maximal binding of [^{35}S]GTPγS attributable to the presence of 0.1 mM carbachol has been reported to be 2−3 mol of nucleotide/mol of muscarinic receptor (Hilf *et al.*, 1989); the corresponding value was at least 1, and probably more, for the carbachol-promoted exchange of [^{35}S]GTPγS for GDP in ventricular membranes from Syrian hamsters (Chidiac and Wells, 1992). In platelet membranes, the maximal amount of [^{3}H]GMP-PNP released by epinephrine or PGE_1 was found to be 1.5−3.2 mol/mol of α_2 receptor and 4−7 mol/mol of PGE_1 receptor (Michel and Lefkowitz, 1982). It therefore appears that G-linked receptors in native membranes activate more than an equal number of G proteins, a stoichiometry that is inconsistent with estimates of $[G]_t/[R]_t$ computed according to the mobile receptor model.

Data from reconstituted systems are similarly difficult to rationalize, but for the opposite reason. G proteins or the limiting subunit thereof typically are present in 5−20-fold molar excess relative to muscarinic receptors; in contrast, the incorporation of [^{35}S]GTPγS relative to the total number of receptors is at least 3-fold less (*i.e.*, 1.5−6-fold excess), in spite of incubation times sufficiently long for the reaction to be at or near completion (Tota *et al.*, 1987; Florio and Sternweis, 1989; Ikegaya *et al.*, 1990). Similar discrepancies emerge from the agonist-stimulated binding of nucleotides to G proteins reconstituted with β-adrenergic receptors (Brandt and Ross, 1986) and μ-opioid receptors (Ueda *et al.*, 1988). Amplification therefore is lower than expected and tends to approximate the levels found in native membranes. The failure of reconstituted receptors to activate all of the available G proteins over time may reflect functionally inactive receptors or incomplete incorporation of the G proteins into the phospholipid vesicles. Alternatively, the allosteric interaction between agonist and nucleotide may involve a more stable oligomeric arrangement than is implied by the mobile receptor model.

Anomalous levels of amplification recall evidence that mobility must be restricted if exchange-based schemes are to account for various functional properties, including binding (Neubig, 1994). Since G proteins typically outnumber receptors in native membranes [*e.g.*, Sternweis and Robishaw (1984), Neubig *et al.* (1985), Vatner *et al.* (1988), Ransnas and Insel (1988a), and Graeser and Neubig (1993)], most cannot be accessible to any particular receptor if the multiple states recognized by agonists derive from ligand-promoted coupling [*e.g.*, Neubig *et al.* (1985) and Lee *et al.* (1986)]. A lack of cross-talk in the binding of agonists to α_2-adrenergic, muscarinic, and opiate receptors in NG108−15 membranes similarly points to compartmentalization or other restrictions that limit the exchange of G proteins between free and coupled pools (Graeser and Neubig, 1993).

The possibility that exchange occurs only within distinct compartments may serve to rationalize the behavior of receptors in native membranes. It seems less satisfactory for reconstituted systems, where receptors and G proteins are likely to be distributed randomly among the phospholipid vesicles. Muscarinic receptors have been found to reveal a dispersion of affinities at concentrations of G proteins that exceed the nominal concentration of the receptor by 5-fold or more [*e.g.*, Florio and Sternweis (1985), Haga *et al.* (1986), Tota *et al.* (1987), and Ikegaya *et al.* (1990)]. Studies in which the receptor was titrated with G protein confirm that the retention of multiple affinities is not due to a limiting concentration of the latter (Haga *et al.*, 1986; Ikegaya *et al.*, 1990).

Although ligand-induced stabilization and destabilization have been well characterized *in vitro*, the role of those processes in transduction remains unclear. Several receptors are known to remain associated with G proteins upon solubilization and purification [*e.g.*, Limbird *et al.* (1980), Cybulsky *et al.* (1981), Senogles *et al.* (1987), Matesic *et al.* (1989, 1991), Poyner *et al.* (1989), and Wreggett and Wells (1995)], and G proteins can remain coupled to receptors in the presence of guanyl nucleotides (Matesic *et al.*, 1989; Poyner *et al.*, 1989). Histaminergic receptors labeled by [^{3}H]histamine in digitonin-solubilized preparations from rat cortex exhibit at least two states of affinity for the ligand; manipulations with GMP-PNP and magnesium have shown that the sites can be made to interconvert from high to low affinity or from low to high affinity and back (Wells and Cybulsky, 1990). The reversible nature of the interconversion supports the notion that the allosteric interactions between agonists and guanyl nucleotides are mediated via receptors and G proteins within a stable hetero-oligomeric array: either the oligomer does not dissociate or dissociated subunits do not exchange prior to reassociation.

Alternatives to the Notion of Ligand-Regulated Coupling. Discrepancies between the present data and Scheme 1 may arise from processes, such as the stepwise dissociation of α and $\beta\gamma$, that are not encompassed by the model. The potential complexity of such schemes is great, even in highly controlled systems, and much of the available data very likely can be described with a sufficient number of parameters. In several respects, however, the nature of the problem suggests that the solution may not lie in a more elaborate model constructed within the same mechanistic context. The properties of Scheme 1 and related proposals are restricted by two related assumptions: first, that multiple states of affinity arise wholly from ligand-regulated equilibria between coupled and uncoupled states; and second, that coupling is strictly heteromeric. An alternative explanation is examined in the accompanying paper, where the data are assessed in terms of cooperative interactions among multiple equivalents of receptor (Chidiac *et al.*, 1997).

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