Effects of Adenyl Nucleotides and Carbachol on Cooperative Interactions among G Proteins†

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Received February 25, 1992; Revised Manuscript Received July 6, 1992

ABSTRACT: Muscarinic agonists and adenyl nucleotides are noncompetitive modulators of sites labeled by [^{35}S]GTPγS in washed cardiac membranes from Syrian golden hamsters. Specific binding of the radioligand and its inhibition by either GTPγS or GDP reveals three states of affinity for guanyl nucleotides. In the absence of adenyl nucleotide, carbachol promotes an apparent interconversion of sites from higher to lower affinity for GDP; the effect recalls that of guanyl nucleotides on the binding of agonists to muscarinic receptors. In the presence of 0.1 mM ATPγS, the binding of [^{35}S]GTPγS is increased at concentrations up to about 50 nM and decreased at higher concentrations. At a radioligand concentration of 160 pM, binding exhibits a bell-shaped dependence on the concentration of both ATPγS and AMP-PNP; with ADP and ATP, there is a second increase in bound [^{35}S]GTPγS at the highest concentrations of adenyl nucleotide. ATPγS and AMP-PNP also modulate the effect of GDP, which itself emerges as a cooperative process: that is, binding of the radioligand in the presence of AMP-PNP exhibits a bell-shaped dependence on the concentration of GDP; moreover, the GDP-dependent increase in bound [^{35}S]GTPγS is enhanced by carbachol. The interactions among GDP, GTPγS, and carbachol can be rationalized quantitatively in terms of a cooperative model involving two sites tentatively identified as G proteins. Both GTPγS and GDP exhibit negative homotropic cooperativity; carbachol enhances the homotropic cooperativity of GDP and induces or enhances positive heterotropic cooperativity between GDP and [^{35}S]GTPγS. An analogous mechanism may underlie the guanyl nucleotide-dependent binding of agonists to muscarinic receptors. The data suggest that the binding properties of G proteins and their associated receptors reflect cooperative effects within heterooligomeric arrays; agonist-induced changes in cooperativity may facilitate the exchange of GTP for bound GDP and thereby constitute the mechanism of G protein activation in vivo.

Some 80% of known hormones and neurotransmitters elicit cellular responses via receptors that act through G proteins[1] (Birnbaumer et al., 1990). The binding of agonists to G protein-linked receptors is subject to the negative allosteric effects of GTP, GDP, and hydrolysis-resistant analogues such as GTPγS, GMP-PNP, and GDPβS (Birnbaumer et al., 1985); similarly, the binding of GDP and possibly other nucleotides to receptor-linked G proteins is reduced by agonists (e.g., Cassel & Selinger, 1978; Michel & Lefkowitz, 1982; Tota et al., 1987; Quist, 1992). The interactions between agonists and guanyl nucleotides are modulated by agents such as magnesium (Northup et al., 1982; Carty et al., 1990) and lithium (Avissar et al., 1988) and by covalent modifications such as ADP-ribosylation of the G protein (Ui, 1984).

Sites labeled by [^{35}S]GTPγS in membranes and reconstituted preparations containing G proteins and receptors exhibit multiple forms or states of affinity differentiated by GDP (Tota et al., 1987; Hilf et al., 1989; Ikegaya et al., 1990); noncompetitive effects of agonists on the binding of GDP appear to involve an interconversion of sites from higher to lower affinity for the nucleotide. G protein-linked receptors similarly exhibit multiple forms or states of affinity differentiated by agonists (De Lean et al., 1980; Sokolovsky et al., 1983; Lee et al., 1986), and guanyl nucleotides promote an apparent interconversion from higher to lower affinity for the agonist. The allosteric interactions between agonists and guanyl nucleotides constitute the mechanistic basis of transduction (Gilman 1987; Birnbaumer et al., 1990). The underlying molecular events remain unresolved, but the apparent reciprocity suggests that a common mechanism determines the affinity of agonists for receptors on the one hand and of guanyl nucleotides for G proteins on the other.

It has been noted periodically that the dispersion of affinities characteristic of G protein-linked receptors may reflect negatively cooperative effects between successive equivalents of the agonist (e.g., Limbird et al., 1975; Sokolovsky et al., 1983; Mattera et al., 1985). The evidence for cooperativity is ambiguous, however, and the dispersion generally is attributed to a mixture of free and G protein-coupled receptors in random and rapid exchange between the two forms (De Lean et al., 1980; Birnbaumer et al., 1990); the allosteric interactions between agonists and guanyl nucleotides are thought to reflect the effect of each ligand on prevailing levels of the receptor–G protein complex, which presumably are increased by the former and decreased by the latter.

Adenyl nucleotides are reported either to be without effect on the binding of agonists (Berrie et al., 1979; Steinberg et al., 1985) or to exhibit GTP-like effects at relatively high concentrations (Rodbell et al., 1971b; Williams & Lefkowitz,

† This investigation was supported by the Medical Research Council of Canada and the Heart and Stroke Foundation of Ontario. P.C. was the recipient of an Ontario Graduate Scholarship. Preliminary reports of this work have been presented at the Third IBRO World Congress of Neuroscience, Montreal, Canada, August 4–9, 1991; and at the Seventh International Symposium on Cellular Endocrinology, Lake Placid, NY, September 26–29, 1991.

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[1] Abbreviations: G protein, trimeric guanyl nucleotide-binding protein comprising α, β, and γ subunits; G_s and G_i, G proteins mediating the stimulation and inhibition of adenylate cyclase; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); AMP-PNP, 5′-adenylyl imidodiphosphate; ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GMP-PNP, 5′-guanylyl imidodiphosphate; [^{35}S]GTPγS, guanosine 5′-*O*-[3-(γ-[^{35}S]thio)triphosphate]; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/92/0431-10908$03.00/0

1977). In studies on membrane preparations, an excess of adenyl nucleotide sometimes is included with labeled or unlabeled guanyl nucleotides in order to minimize nonspecific binding (e.g., Michel & Lefkowitz, 1982; Wong et al., 1990) or enzymatic degradation (Salomon & Rodbell, 1975; Lad et al., 1977). It is assumed in such studies that adenyl nucleotides have no appreciable effect on the interactions between guanyl nucleotides and G proteins, although ATP recently has been reported to increase the binding of [^{3}H]GMP-PNP (Ho et al., 1991).

In contrast to receptor-specific ligands, the selectivity of labeled guanyl nucleotides is often in doubt. In cardiac membranes, for example, potential binding sites for guanyl nucleotides include a variety of G proteins (Birnbaumer et al., 1990), smaller GTP-binding proteins such as the products of *ras* oncogenes (Gibbs & Marshall, 1989), and perhaps nonspecific nucleotidases (Salomon & Rodbell, 1975). That uncertainty notwithstanding, agonist-induced changes in the binding of guanyl nucleotides presumably are restricted to those G proteins linked to the relevant receptor. Comparable selectivity is not necessarily expected of inorganic cations and other agents that modify binding in a less specific manner.

The present results suggest that adenyl nucleotides and the muscarinic agonist carbachol are allosteric modulators of G proteins labeled by [^{35}S]GTPγS in myocardial membranes from Syrian hamsters; moreover, a dispersion of affinities revealed by GDP and GTPγS can be attributed to cooperative effects between successive equivalents of nucleotide. The results suggest that the binding properties of G proteins and their associated receptors are a manifestation of cooperative interactions within oligomeric arrays.

MATERIALS AND METHODS

Chemicals. [^{35}S]GTPγS (1100–1300 Ci/mmol) was purchased from New England Nuclear. GDP, unlabeled GTPγS, AMP-PNP, ATPγS, cyclic AMP, ADP, and HEPES were purchased from Boehringer Mannheim. Adenosine was purchased from Research Biochemicals Inc. Other nucleotides, dithiothreitol, bacitracin, and muscarinic ligands were purchased from Sigma Chemical Co. Other chemicals were of reagent grade or better.

Preparation of Tissue. Adult male Syrian golden hamsters were obtained from Harlan Sprague-Dawley, Indianapolis, IN. Left ventricles including the interventricular septum were homogenized by means of a Brinkman Polytron, and the membranes were washed with a buffer containing EDTA and bacitracin; further details have been described elsewhere (Chidiac et al., 1991). Pellets of washed membranes were stored at −75 °C until required for the binding assays.

A washing procedure was included to remove endogenous nucleotides, particularly GDP, that otherwise could be expected to complicate the interpretation of the data (e.g., Lad et al., 1980). Muscarinic receptors and either G proteins or their α subunits reportedly are released from membranes under some conditions (Ho et al., 1991; Lynch et al., 1986; Ransnas & Insel, 1988; Mulligan et al., 1988; Iyengar et al., 1988), but no loss was discernible under those of the present investigation. The maximal specific binding of *N*-[^{3}H]-methylscopolamine to muscarinic receptors in washed membranes from hamster myocardium [136 ± 7 pmol/g of protein (Chidiac et al., 1991); 155 ± 5 pmol/g of protein in the present investigation] is similar or the same as that found that unwashed membranes [81–174 pmol/g of protein (Wong et al., 1986)]. Also, there is no deleterious effect on those G proteins responsible for the nucleotide-sensitive, high-affinity binding of muscarinic agonists or on those that mediate either the stimulation or the inhibition of adenylate cyclase (Chidiac et al., 1991).

The effective removal of GDP is suggested by the observation that the sites of highest affinity for muscarinic agonists can account for as much as 70% of the capacity for *N*-[^{3}H]-methylscopolamine when binding to washed membranes is assayed in appropriate buffers; more extensive washing is without further effect.[2] Also, the time-dependent accumulation of cyclic [^{32}P]AMP in the absence or presence of carbachol is linear for at least 15 min and intersects the ordinate at the origin; measurements as early as 20 s show no evidence of any deviation from linearity (Chidiac et al., 1991). Finally, the results of the present investigation indicate that GDP is required for an effect of carbachol on the specific binding of [^{35}S]GTPγS; the agonist has no discernible effect on binding of the radioligand alone, and the level of endogenous GDP in washed membranes thus appears to be negligible.

Binding of [^{35}S]GTPγS. A Potter-Elvehjem tissue blender was used to resuspend thawed pellets in buffer containing HEPES (10 mM), NaCl (100 mM), $MgCl_2$ (5 mM), dithiothreitol (1 mM), phenylmethanesulfonyl fluoride (0.1 mM), and EGTA (1 mM) at pH 7.4. Protein was assayed according to the procedure of Lowry et al. (1951), using bovine serum albumin as the standard, and the membranes were diluted to yield a protein concentration of 0.5 mg/mL. Binding was measured after incubation for 2.5 h at 30 °C, and procedures otherwise were as described by Wong et al. (1986); bound and free radioligand were separated by microcentrifugation. [^{35}S]GTPγS was diluted isotopically to a specific activity of 12–26 Ci/mmol for experiments at graded concentrations of the radioligand and otherwise was used as purchased. Nonspecific binding was taken as total binding in the presence of 0.1 mM unlabeled GTPγS. For the experiments shown in Figure 4, tissue was preincubated with GDP with or without carbachol (2 mM) for 15 min at 30 °C; the radioligand then was added either alone or together with adenyl nucleotide, and the mixture was incubated for a further 2.5 h. The samples were processed as described above. All assays were performed in quadruplicate; the radioactivity of each sample was measured twice, and the eight values were averaged to obtain the mean (±SEM) for subsequent analyses. The standard error of the mean was less than 1.5% for approximately two-thirds of the values.

Control experiments indicated that guanyl nucleotides were at or near equilibrium as regards their interaction with the sites labeled by [^{35}S]GTPγS. Binding of the radioligand alone and together with selected concentrations of GDP became independent of time within 2 h and remained stable for at least 2 h thereafter. Also, most if not all specifically bound [^{35}S]GTPγS was found to dissociate following equilibration at a total radioligand concentration of 160 pM; the time course is not a single exponential, but specific binding was reduced to about 20% of the equilibrium level by 5 h. Binding of guanyl nucleotides is not necessarily reversible on the time scale of typical assays, and the dissociation of [^{35}S]GTPγS from G proteins has been found to be immeasurably slow under some conditions (e.g., Northup et al., 1982; Bokoch et al., 1984; Higashijima et al., 1987). The time dependence of binding under the conditions of the present studies will be described elsewhere.[3]

Analysis of Data. Empirical descriptions of binding at graded concentrations of an unlabeled ligand were obtained according to eq 1, in which B_{obsd} is the total binding of [^{35}S]-

[2] Marty A. Green and J.W.W., unpublished observations.
[3] P.C. and J.W.W., unpublished observations.

GTPγS (disintegrations per minute per milliliter) and $[X]_t$ is the total concentration of the unlabeled ligand. The parameters $B_{[X]=0}$ and $B_{[X]\to\infty}$ represent the asymptotic values of B_{obsd} when $[X]_t = 0$ and as $[X]_t \to \infty$; K_j is the value of $[X]_t$ corresponding to a half-maximal signal at the fraction F'_j of the net change in B_{obsd} ($\sum_{j=1}^{n} F'_j = 1$).

$$B_{obsd} = B_{[X]\to\infty} + (B_{[X]=0} - B_{[X]\to\infty}) \sum_{j=1}^{n} \frac{F'_j K_j}{K_j + [X]_t} \quad (1)$$

Individual values of F'_j are between 0 and 1 when the first derivative of eq 1 is negative at all values of $[X]_t$; when $f'(x)$ traverses zero ($n \geq 2$), at least one value of F'_j will be outside this range (i.e., $F'_j < 0$ or $F'_j > 1$). The latter behavior is characteristic of positive cooperativity.

Equation 1 is a rational function; it therefore is equivalent to either of the mechanistic models described below, provided that the free and total concentrations of all ligands are essentially equal. In the present investigation, specific binding accounted for up to 25% of total [^{35}S]GTPγS at the concentration used to characterize the behavior of unlabeled ligands (i.e., 160 pM). The occurrence of appreciable depletion can be expected to compromise the explicit relationships that otherwise exist between the parameters of eq 1 and those of mechanistic models.

Mechanistic descriptions of the data were obtained by fitting eq 2 to estimates of total binding (B_{obsd}) taken as measured (disintegrations per minute per milliliter). The quantity $[P]_b$ represents the specific binding of [^{35}S]GTPγS at a total concentration $[P]_t$; SA is the specific radioactivity (curies per millimole), and NS is the fraction of unbound [^{35}S]GTPγS that ultimately appears as nonspecific binding.

$$B_{obsd} = \{[P]_b + NS([P]_t - [P]_b)\}SA(2.22 \times 10^{12}) \quad (2)$$

Values of $[P]_b$ were calculated according to Schemes I and II, in which P and A represent [^{35}S]GTPγS and an unlabeled nucleotide, respectively; affinities are represented throughout as the equilibrium dissociation constant (K). Both P and A entered into the various equations as total concentration rather than free, and the solutions were obtained numerically as described elsewhere (Wells, 1992).

Scheme I describes a multisite model in which binding is to n classes of distinct and mutually independent sites (R_j, where $j = 1, 2, \ldots, n$). When P and A represent [^{35}S]GTPγS and unlabeled GTPγS, respectively, both ligands are assumed to bind with equal affinity to the sites of type j (i.e., $K_j = K_{Aj} \equiv K_{Pj}$). Total specific binding of the probe ($[P]_b$) is defined by eq 3, and the value was obtained as the appropriate root

$$[P]_b = [PR_1] + [PR_2] + \ldots + [PR_n] \quad (3)$$

of a quartic polynomial (i.e., eq 67 in Wells, 1992). Capacity was optimized as the total concentration of all sites (i.e., $[R]_t = \sum_{j=1}^{n} [R_j]_t$, where $[R_j]_t = [R_j] + \sum_{j=1}^{n} ([PR_j] + [AR_j])$) and that fraction F_j corresponding to sites of type j (i.e., $F_j = [R_j]_t/[R]_t$).

Scheme II describes a hybrid model that comprises a heterogeneous mixture of bivalent and monovalent components designated as R and S, respectively; there is no relationship between the elements of each class, which are defined as mutually independent and noninterconverting. The bivalent component R represents a cooperative system, in that occupancy of one site affects the affinity of the second. If R represents a dimer of G proteins [i.e., $(\alpha\beta\gamma)_2$], it is implicit that there is no dissociation into free G proteins or further into α and βγ subunits. An important exception to this restriction arises when individual elements dissociate and reassociate without exchanging partners; in that event, the concentration of free G protein or subunits thereof is irrelevant, and the formulation of the model is unchanged from that used here. The parameters K_{PR}, K_{RP}, K_{AR}, and K_{RA} represent microscopic dissociation constants; a, b, and c represent the cooperativity factors for the change in free energy of binding associated with successive levels of occupancy. Asymmetry is not detectable with the present data, and it therefore was assumed that all ligands bound with equal affinity to either site of the vacant dimer (i.e., $K_{PR} = K_{RP}$ and $K_{AR} = K_{RA}$); accordingly, the fitting procedure was programmed to optimize the values of K_{PR}, K_{AR}, K_{PS}, K_{AS}, a, b, and c. The labeled and unlabeled analogues of GTPγS were assumed to be functionally identical, as in Scheme I; when both were present, the optimized parameters were K_{PR}, K_{PS}, and a (i.e., $K_{PR} = K_{AR}$, $K_{PS} = K_{AS}$, and $a = b \equiv c$). In all analyses, the set of parameters also included $[R]_t + [S]_t$ and F_D, where F_D equals the fraction $[R]_t/([R]_t + [S]_t)$; individual values of $[R]_t$ and $[S]_t$ were calculated from $[R]_t + [S]_t$ and F_D as required. Since the stoichiometry of binding is 2:1 for R and 1:1 for S, a value of x for F_D corresponds to a value of $2x/(x + 1)$ for the fraction of binding sites associated with R. Total specific binding of the probe in Scheme II is described by

$$[P]_b = [PR] + [RP] + 2[PRP] + [PRA] + [ARP] + [PS] \quad (4)$$

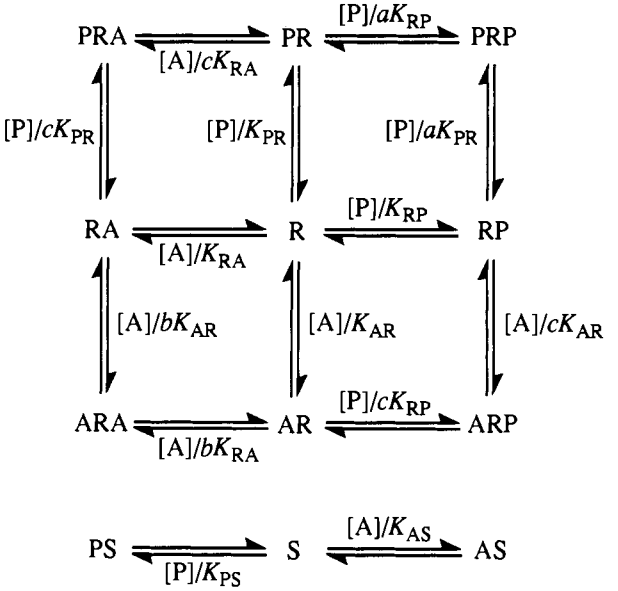
The concentration of each complex was calculated from $[R]_t$, $[S]_t$, and the free concentrations of P and A; the latter were obtained by solving the set of implicit equations for all reactants [eqs 171–173 in Wells (1992)].

All parameters were estimated by nonlinear regression as described previously (Wong et al., 1986; Wells, 1992). Values at successive iterations of the fitting procedure were adjusted according to the algorithm of Marquardt (1963). Most analyses involved at least some parameters shared among multiple sets of data acquired in several experiments. Estimates of the absolute signal in eq 1 (i.e., $B_{[X]=0}$ and $B_{[X]\to\infty}$), in eqs 2 and 3 (i.e., NS and $[R]_t$), and in eqs 2 and 4 (i.e., NS and $[R]_t + [S]_t$) were unique to individual sets of data

Scheme I

$$PR_j \underset{[P]/K_{Pj}}{\rightleftharpoons} R_j \overset{[A]/K_{Aj}}{\rightleftharpoons} AR_j$$

Scheme II



except as described below; individual values were averaged to obtain the mean (±SEM). Binding at graded concentrations of GDP was measured routinely in the absence and presence of carbachol (2 mM), and the assays were performed in parallel under otherwise identical conditions; the agonist was found to be without significant effect on either $[R]_t + [S]_t$ or NS in eqs 2 and 4, and single values of those parameters therefore were assigned in common to both sets of data. All other parameters generally were common to data from three or more experiments, and the assignments are described in the legends to the figures and tables. Fitted parametric values are presented together with the errors as estimated from the diagonal elements of the covariance matrix; the latter reflect the range over which the global sum of squares is insensitive to the value of the parameter. NS was defined throughout as the binding of [^{35}S]GTPγS in the presence of 0.1 mM unlabeled GTPγS.

Statistical procedures were carried out as described previously (Wong et al., 1986). Standard errors on measurements of binding tended to be a constant percentage of the mean, and the data were weighted accordingly. Weighted residuals were of comparable magnitude within single sets of data, and multiple sets of data made comparable contributions to the total sum of squares from simultaneous analyses; accordingly, statistical assessments were not dominated by the residuals from one experiment or group of experiments. The effect of more or fewer parameters on the sum of squares of weighted residuals was tested for significance by means of the F statistic. Mean parametric values were compared using the t statistic.

Data from replicated experiments have been presented with reference to a single fitted curve in Figures 1B and 4, which illustrate the results of simultaneous analyses according to Schemes I and II. To obtain the values plotted on the ordinate, estimates of B_{obsd} were adjusted according to

$$B'_{obsd} = B_{obsd}\frac{f(\bar{\mathbf{x}}_i,\bar{\mathbf{a}})}{f(\mathbf{x}_i,\mathbf{a})} \quad (5)$$

The function f represents eq 2 and either 3 or 4. The vectors $\mathbf{x}_i$ and $\mathbf{a}$ represent the independent variables at point i and the fitted parameters for the set of data under consideration; $\bar{\mathbf{x}}_i$ and $\bar{\mathbf{a}}$ are the corresponding vectors in which values that differ from experiment to experiment have been replaced by the means for all experiments associated with the fitted curve. Individual values of B'_{obsd} at the same $\mathbf{x}_i$ were plotted separately (Figure 1B) or were averaged to obtain the mean and standard error (Figure 4).

RESULTS

Binding of [^{35}S]GTPγS. Graded concentrations of labeled and unlabeled GTPγS yielded the binding profiles illustrated in Figure 1, which includes a comparison of data acquired in the absence of adenyl nucleotide and in the presence of 0.1 mM ATPγS. The fitted curves were obtained by assuming up to three classes of distinct and noninterconverting sites (Scheme I, eq 3), as appropriate, and the parametric values are listed in Table I. ATPγS was found to reduce nonspecific binding relative to that in parallel controls performed under otherwise identical conditions (inset, Figure 1A); specific binding of [^{35}S]GTPγS was increased at lower concentrations of the radioligand and decreased at higher concentrations, with the result that the curves intersect at a radioligand concentration of about 50 nM (Figure 1A). The same intersection occurs with unlabeled GTPγS as the independent variable (Figure 1B).

In the absence of adenyl nucleotide, [^{35}S]GTPγS revealed two classes of sites in terms of eq 3. The value of K_1 is well-

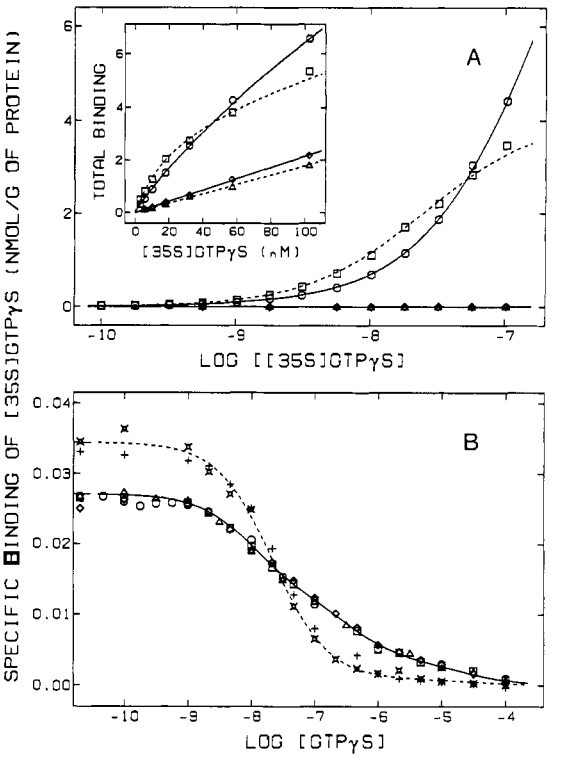


FIGURE 1: Effect of ATPγS on the binding of labeled and unlabeled GTPγS. Total binding was measured following incubation of the membranes with graded concentrations of [^{35}S]GTPγS (A) or the unlabeled analogue at a constant concentration of [^{35}S]GTPγS (B). The lines represent best fits of eqs 2 and 3 to the combined data obtained from three or four experiments performed under each set of conditions (solid lines, no ATPγS; dashed lines, 0.1 mM ATPγS); further details are described in the text and in the footnote to Table I. The data shown in panel A are from one of three experiments included in the analysis; binding was measured concomitantly with the radioligand alone (○), in the presence of 0.1 mM GTPγS (◇), in the presence of 0.1 mM ATPγS (□), and in the presence of both GTPγS and ATPγS (△). Parametric values corresponding to the data from all three experiments are listed in Table I; the values of $[R_i]_t$ and NS for the data shown in the figure are as follows: no ATPγS (n = 2), $[R_1]_t$ = 57 pM, $[R_2]_t$ = 6.1 ± 0.5 nM, and NS = 0.0213 ± 0.0004; 0.1 mM ATPγS (n = 1), $[R_1]_t$ = 2.1 ± 0.1 nM and NS = 0.0177 ± 0.0008. Values plotted on the ordinate in the outer frame represent total binding less the fitted estimate of nonspecific binding; total binding is shown in the inset, where data at radioligand concentrations below 2 nM have been omitted for clarity. The data shown in panel B are from separate experiments performed in the absence of adenyl nucleotide (○, □, ◇, △) and in the presence of 0.1 mM ATPγS (+, ⊗). The former represent all of the data included in the analysis, and the mean concentration of [^{35}S]GTPγS was 170 ± 5 pM. The latter represent two out of three experiments included in the analysis (159 and 167 pM [^{35}S]GTPγS); the third experiment was performed at a radioligand concentration of 10.4 nM, and the fit is comparable to that illustrated in the figure. To obtain the values plotted on the ordinate, estimates of B_{obsd} were adjusted according to eq 5; specific binding was taken as B'_{obsd} less the value of $f(\bar{\mathbf{x}}_i,\bar{\mathbf{a}})$ as [A] → ∞. Points at the lower end of the abscissa represent binding in the absence of unlabeled GTPγS, and log [GTPγS] was taken arbitrarily as −15.

defined by the data; K_2 exceeds the highest concentration of the radioligand, and it therefore is correlated with $[R_2]_t$, but the fitted value nevertheless corresponds to a clear minimum in the sum of squares. Graded concentrations of unlabeled GTPγS revealed three classes of sites, the weakest of which is not detected at the concentrations of [^{35}S]GTPγS that are practicable in experiments at graded concentrations of the radioligand. The two methods yielded comparable estimates of K_2, but K_1 is somewhat higher with unlabeled GTPγS as the independent variable (Table I). ATPγS reduced the number of classes of sites by one in each case. A single class was sufficient to describe binding at graded concentrations of

Table I: Effect of ATPγS on the Binding of GTPγS in Terms of Scheme I[a]

variable ligand	ATPγS (mM)	affinity			capacity (nmol/g of protein)		
		log K_1	log K_2	log K_3	$R_{1,t}$	$R_{2,t}$	$R_{3,t}$
[^{35}S]GTPγS	0.0	−8.71 ± 0.05	−6.73 ± 0.21		0.11 ± 0.01	12 ± 1	
[^{35}S]GTPγS	0.1	−7.62 ± 0.02			5.0 ± 0.9		
GTPγS	0.0	−8.07 ± 0.05	−6.52 ± 0.16	−4.63 ± 0.13	0.78 ± 0.18	19 ± 4	486 ± 113
GTPγS	0.1	−7.74 ± 0.03		−4.56 ± 0.46	4.6 ± 0.4		171 ± 15

[a] For each set of conditions described in the table, data from three or four experiments were analyzed simultaneously according to eq 2, with $[P]_b$ computed according to Scheme I (eq 3). In each experiment with [^{35}S]GTPγS as the variable ligand, binding was measured with the radioligand alone and in the presence of 0.1 mM ATPγS, 0.1 mM GTPγS, and 0.1 mM ATPγS plus 0.1 mM GTPγS; when the variable ligand was unlabeled GTPγS, measurements with and without ATPγS were performed in separate experiments. Within each analysis, parameters were shared among multiple sets of data as follows: single values of K_j and F_j for all data and separate values of $[R]_t$ and NS for the data from each experiment; accordingly, single values of $[R]_t$ and NS were common to the two sets of data acquired with and without unlabeled GTPγS in each experiment at graded concentrations of the radioligand. The fitted values of K_j are listed in the table. Fitted values of $[R]_t$ were normalized per gram of protein, and the value of F_j was used to obtain $R_{j,t}$ for each experiment; individual values then were averaged to obtain the means (±SEM) listed in the table. The fitted values of F_j are as follows: [^{35}S]GTPγS without ATPγS, $F_2 = 0.9911 \pm 0.0045$; GTPγS without ATPγS, $F_2 = 0.0369 \pm 0.0004$ and $F_3 = 0.962 \pm 0.001$; GTPγS with ATPγS, $F_3 = 0.974 \pm 0.035$. The data and fitted curves are illustrated in Figure 1; further details are described in the legend to Figure 1 and in the text.

the radioligand; two classes were required for unlabeled GTPγS, although the sites of lower affinity accounted for only 2.8% of the specific signal (e.g., F'_2 in eq 1 when $n = 2$).

In terms of eq 3, the intersection brought about by ATPγS reflects decreases in both $R_{2,t}$ and $R_{3,t}$ on the one hand and an increase in $R_{1,t}$ on the other (Table I). Any change in capacity implies that ATPγS and GTPγS do not compete in the manner defined by the multisite model, although an apparent decrease might occur if ATPγS were to exchange slowly relative to the radioligand. An apparent increase is inconsistent with any scheme for mutually exclusive binding, reversible or otherwise, unless the system were to cycle in a manner analogous to that described by Katz and Thesleff (1957). It follows that adenyl nucleotides appear to influence the binding of GTPγS in an allosteric manner.

Dose-Dependent Effects of Adenyl Nucleotides on the Binding of [^{35}S]GTPγS. The noncompetitive behavior of adenyl nucleotides is illustrated further by their concentration-dependent effects on the binding of [^{35}S]GTPγS at a total concentration of approximately 160 pM. ATPγS yielded the bell-shaped pattern illustrated in Figure 2A; lower concentrations increased binding, and higher concentrations inhibited to a level indistinguishable from that measured in the presence of 0.1 mM unlabeled GTPγS. AMP-PNP behaved similarly (Figure 2B), although the increase was more pronounced, and the inhibition was incomplete at the highest concentration used. A more complex pattern emerged with ADP (Figure 2C) and ATP (Figure 2D); with either compound, an initial increase in the binding of [^{35}S]GTPγS was followed successively by a decrease and then an increase.

Increased binding of [^{35}S]GTPγS implies that adenyl nucleotides act in a positively cooperative manner, provided that the system is at thermodynamic equilibrium. This condition was tested in an experiment similar to those represented in Figure 2D, but in which binding was measured once after incubation for 2.5 h and again after 3.25 h; the concentration of ATP was varied over the range from 0.1 μM to 3.2 mM. The two sets of data were indistinguishable, and there was excellent agreement with the results illustrated in Figure 2D. Binding therefore becomes independent of time within 2.5 h at each concentration of ATP.

The fitted curves in Figure 2 were obtained empirically according to eq 1, which is readily identified with the multisite model (Scheme I) but also is formally indistinguishable from the cooperative model depicted in Scheme II. Both models yield rational functions wherein the degree of the polynomial in the denominator reflects the minimum number of sites through which the adenyl nucleotide affects binding of the radioligand (e.g., n in eq 1). The number of sites is defined partly by the number of inflections discernible in the binding pattern, and the required value of n with eq 1 is 2 for ATPγS and AMP-PNP and 3 for ATP and ADP; if binding eventually returns to the level observed in the presence of 0.1 mM unlabeled GTPγS, at least one additional site is implied for AMP-PNP, ATP, and ADP.

ADPβS increased the binding of [^{35}S]GTPγS but showed no inhibitory effect at concentrations up to 0.32 mM (Figure 3). The pyrimidines ITP and UTP were strictly inhibitory, with half-maximal effects at 0.57 and 37 μM, respectively. Adenosine had little or no effect at concentrations up to 1 mM. The binding of the radioligand similarly was unaffected by 10 μM cyclic AMP or by AMP at concentrations up to 0.1 mM. Adenosine and cyclic AMP also had no effect on the signal at intermediate concentrations of ATP or ATPγS, respectively: in the presence of 10 μM ATP, binding was insensitive to adenosine at concentrations up to 1 mM, and in the presence of 10 μM ATPγS, binding was insensitive to cyclic AMP at concentrations up to 10 mM. Finally, the binding of [^{35}S]GTPγS at graded concentrations of ATPγS was unaffected by the muscarinic agonist carbachol at a concentration of 2 mM.

Effects of Adenyl Nucleotides and Carbachol on the Binding of GDP. At a radioligand concentration of approximately 160 pM, GDP affected binding via at least two sites under all conditions tested. In the absence of adenyl nucleotide, an increase of at least 50 000-fold in the concentration of GDP was required to reduce the specific binding of the radioligand from 90% to 10% of that observed in the absence of the unlabeled ligand (Figure 4A). Three classes of sites are required for agreement between eq 1 and the data. A saturating concentration of carbachol had little effect on the nature or the breadth of the dispersion, which still required three classes of sites, but the value of log IC_{50} was increased from −6.13 to −5.17. At a GDP concentration of 10 μM and a radioligand concentration of 160 pM, the carbachol-dependent increase in binding could be described by assuming a single class of sites for the agonist (i.e., $n = 1$ in eq 1); the half-maximal increase occurred at 6.5 μM carbachol (log $EC_{50} = -5.19 \pm 0.18$), and the effect was inhibited fully by the muscarinic antagonist atropine at a concentration of 0.1 μM. Three muscarinic agonists were compared for their effect at a concentration of 1 mM in the presence of 136 pM [^{35}S]-GTPγS and 10 μM GDP; the increase with oxotremorine-M was 40% of that obtained with carbachol, and the increase with pilocarpine was 78%.

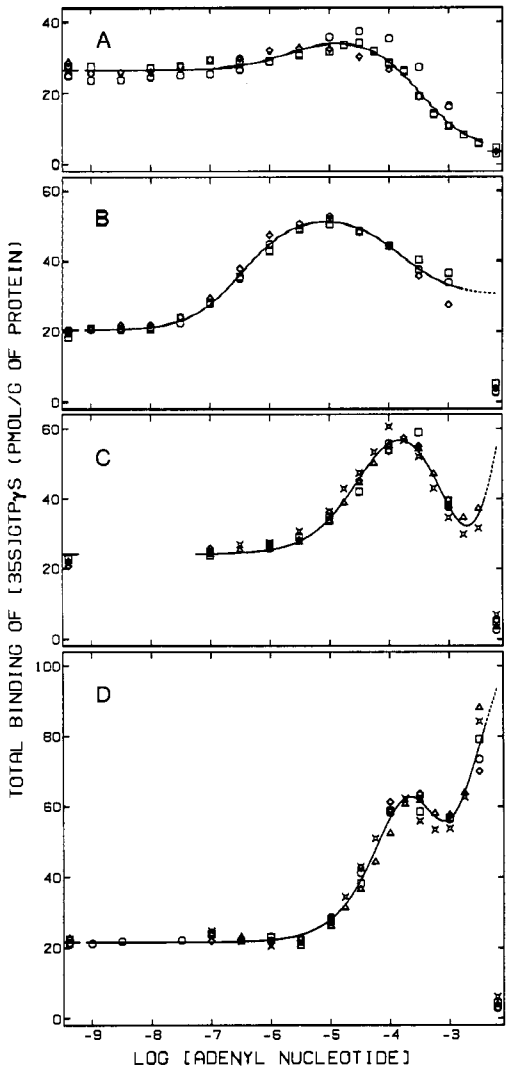


FIGURE 2: Effects of adenyl nucleotides on the binding of [^{35}S]-GTPγS. Total binding was measured following incubation of the membranes with the radioligand (165 ± 3 pM) and graded concentrations of ATPγS (A), AMP-PNP (B), ADP (C), or ATP (D). Different symbols denote data from different experiments. The lines represent best fits of eq 1 to the combined data shown in each panel; single values of K_j and F'_j were common to all of the data, and separate values of $B_{[X]=0}$ and $B_{[X]\to\infty}$ were assigned to the data from each experiment. Values plotted on the ordinate have been normalized to the mean values of $B_{[X]=0}$ and $B_{[X]\to\infty}$ according to eq 5. Points at the lower and upper ends of the abscissa represent binding in the absence of unlabeled ligand and in the presence of 0.1 mM unlabeled GTPγS, respectively; the latter were included at the arbitrary concentration of 1 M in the analysis for ATPγS (panel A) but otherwise were omitted (panels B–D). The parametric values obtained by regression are as follows: panel A ($n = 2$), $\log K_1 = -5.65 \pm 0.26$, $\log K_2 = -3.47 \pm 0.06$, $F'_2 = 1.43 \pm 0.07$; panel B ($n = 2$), $\log K_1 = -6.46 \pm 0.04$, $\log K_2 = -3.82 \pm 0.10$, $F'_2 = -2.41 \pm 0.41$; panel C ($n = 3$), $\log K_1 = -4.41 \pm 0.07$, $\log K_2 = -2.77 \pm 0.68$, $\log K_3 = -2.06 \pm 2.21$, $F'_2 = -1.15 \pm 7.17$, $F'_3 = 1.81 \pm 6.06$; panel D ($n = 3$), $\log K_1 = -3.57 \pm 0.70$, $\log K_2 = -3.44 \pm 1.71$, $\log K_3 = -2.93 \pm 0.91$, $F'_2 = -14 \pm 196$, $F'_3 = 3.75 \pm 14.33$. Errors that exceed the corresponding parametric value reflect uncertainty over the asymptotic level of binding at high concentrations of ADP and ATP.

In the presence of 0.1 mM ATPγS, binding of the radioligand was increased in a manner consistent with the effect of the adenyl nucleotide shown in Figure 1 and Figure 2A. The binding profile of GDP was steeper but otherwise similar to that observed in the absence of ATPγS (Figure 4B). An increase of less than 600-fold in the concentration of GDP was sufficient to reduce the specific binding of [^{35}S]-GTPγS from 90% to 10%, and only two classes of sites are required for agreement with eq 1. Carbachol increased the value of log IC_{50} only slightly, from –6.27 to –6.14. Binding

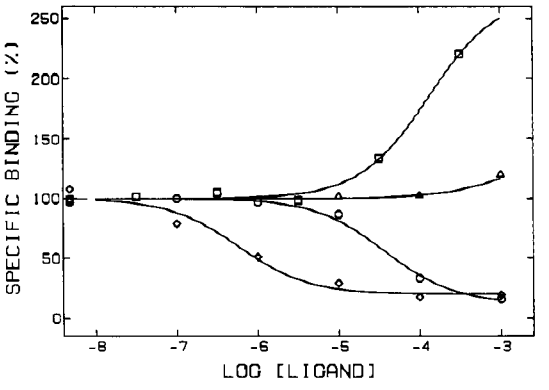


FIGURE 3: Effects of purine and pyrimidine derivatives on the binding of [^{35}S]GTPγS. Total binding was measured following incubation of the membranes with the radioligand (160 ± 1 pM) and graded concentrations of ADPβS (□), ITP (◇), UTP (○), or adenosine (△). The lines represent the best fits of eq 1 ($n = 1$) to the data from individual experiments. Values plotted on the ordinate have been normalized to the fitted values of $B_{[X]=0}$ taken as 100% and to the estimates of binding in the presence of 0.1 mM GTPγS taken as 0%; the latter were omitted from the analyses.

of the radioligand approximately doubled in the presence of 0.1 mM AMP-PNP, in agreement with the data illustrated in Figure 2B, but the effect of GDP differed from the strictly inhibitory behavior observed under other conditions. Concentrations of GDP up to about 1 μM caused a dose-dependent increase in binding beyond that effected by the adenyl nucleotide, while higher concentrations were inhibitory; carbachol increased both the stimulatory effect of GDP and the concentration of GDP required to achieve inhibition (Figure 4C).

The effect of carbachol on the binding of [^{35}S]GTPγS and GDP is illustrated further in Figure 5, where the data represent the difference between the two fitted curves in each frame of Figure 4; the analyses were performed according to Scheme II as described below. In the absence of adenyl nucleotide, the maximal increase in bound radioligand was approximately 4.8 pmol/g of protein at a GDP concentration of about 3.2 μM; the increase in the presence of ATPγS was 2.0 pmol/g of protein at 1.3 μM GDP, and that in the presence of AMP-PNP was 17 pmol/g of protein at 7.3 μM GDP. Carbachol was without effect on binding in the absence of GDP. As estimated from the fitted asymptotes in Figure 4, specific binding in the absence of GDP was increased from 19 to 26 pmol/g of protein upon the inclusion of ATPγS and to 40 pmol/g of protein upon the inclusion of AMP-PNP. The carbachol-dependent increase at the optimal concentration of GDP in the absence of adenyl nucleotide thus corresponds to 26% of specific binding in the absence of GDP; the corresponding values for the increase in the presence of adenyl nucleotide are 7.8% with ATPγS and 43% with AMP-PNP (Figure 5A). If the increase is expressed relative to total specific binding in the presence of carbachol at the same concentration of GDP, the maximal values are 42% in the absence of adenyl nucleotide, 34% in the presence of ATPγS, and 45% in the presence of AMP-PNP (Figure 5B).

Experiments similar to those illustrated in Figure 4A were carried out at concentrations of [^{35}S]GTPγS between 3.7 and 38 nM, in part to examine the selectivity of the radioligand and in part to estimate the minimal ratio of guanyl nucleotide binding sites to muscarinic receptors. The increase effected by carbachol in the binding of [^{35}S]GTPγS exhibited a maximum with respect to both GDP and the radioligand; at 1–10 μM GDP, however, the change always exceeded that of 4.8 pmol/g of protein observed at 3.2 μM GDP and 160 pM [^{35}S]GTPγS. The relative change was smaller, and agonist-

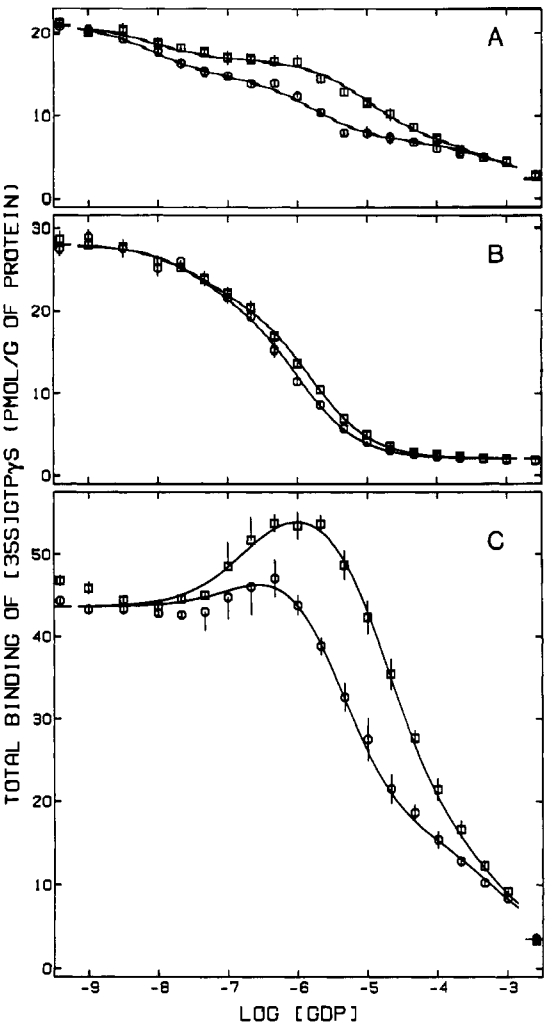


FIGURE 4: Effects of adenyl nucleotides and carbachol on the binding of GDP. Total binding was measured following incubation of the membranes with the radioligand and graded concentrations of GDP in the absence of agonist (○) and in the presence of 2 mM carbachol (□); each experiment was performed three times in the absence of adenyl nucleotide (A), in the presence of 0.1 mM ATPγS (B), and in the presence of 0.1 mM AMP-PNP (C). The lines represent the best fit of eqs 2 and 4 to the 18 sets of data summarized in the figure plus seven sets acquired at graded concentrations of unlabeled GTPγS (i.e., Figure 1B); further details are described in the text and in the footnotes to Table II. Values of B_{obsd} were adjusted according to eq 5, with the final value of $[P]_t$ taken throughout as the mean for the nine experiments shown (154 ± 6 pM); individual values of B'_{obsd} at the same concentration of GDP were averaged to obtain the means (±SEM) plotted on the ordinate. Points at the lower and upper ends of the abscissa represent binding in the absence of GDP and in the presence of 0.1 mM unlabeled GTPγS, respectively; both were included in the analysis.

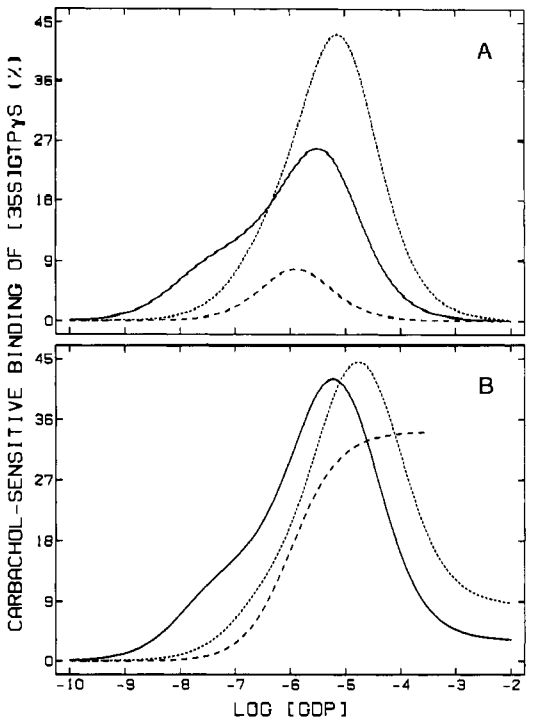


FIGURE 5: Difference curves for the effect of carbachol on the binding of GDP. Values plotted on the ordinate represent the difference between the fitted curves for data acquired in the absence and presence of carbachol in each panel of Figure 4. The agonist-dependent increase in bound [^{35}S]GTPγS at each concentration of GDP is shown as a percentage of specific binding in the absence of GDP (A) and as a percentage of specific binding in the presence of carbachol at the same concentration of GDP (B). Different lines denote binding in the absence of adenyl nucleotide (—, cf. Figure 4A), in the presence of 0.1 mM ATPγS (- - -, cf. Figure 4B), and in the presence of 0.1 mM AMP-PNP (..., cf. Figure 4C).

sensitive binding at 38 nM [^{35}S]GTPγS represented less than 6% of specific binding in the absence of GDP (cf. Figure 5A). It follows that carbachol-sensitive sites were labeled selectivity at subnanomolar concentrations of the radioligand, while carbachol-insensitive sites dominated the signal at higher concentrations. Estimates of the net change effected by carbachol are accompanied by relatively large error at higher concentrations of the radioligand, but the largest increase in bound [^{35}S]GTPγS was 80–130 pmol/g of protein. Maximal specific binding of the muscarinic antagonist *N*-[^{3}H]-methylscopolamine was 155 ± 5 pmol/g of protein in the same preparation.[3] Since the concentration of GDP was sufficient to reduce the specific binding of [^{35}S]GTPγS by 40–60% in the absence of carbachol, it appears that at least 1 equiv of G protein is linked to each muscarinic binding site.

Analysis of Guanyl Nucleotide Binding According to a Cooperative Model. If [^{35}S]GTPγS and GDP were at equilibrium with respect to their interaction with the labeled sites, the GDP-dependent increase illustrated in Figure 4C is indicative of cooperative interactions between the two ligands. The binding patterns therefore were assessed for their agreement with Scheme II (eq 4). The analyses involved 25 sets of data acquired under various conditions at graded concentrations of unlabeled GTPγS (Figure 1B) or GDP (Figure 4): in the case of GTPγS, the data represented binding in the absence and presence of ATPγS, and in the case of GDP, the data represented binding with and without carbachol in the absence of adenyl nucleotide, in the presence of ATPγS, and in the presence of AMP-PNP. Controls indicated that the muscarinic agonist carbachol was without effect on the inhibitory behavior of unlabeled GTPγS; indeed, no effect of carbachol was observed under any conditions in the absence of GDP.

Preliminary analyses in terms of eq 4 demonstrated that the dimer alone (i.e., $F_D = 1$) is sufficient to describe binding in the presence of ATPγS but not that in the absence of adenyl nucleotide or in the presence of AMP-PNP; lack of agreement is indicated by marked and systematic deviations between the data and the fitted curves (not shown). The discrepancies were resolved by expanding the model to include a separate class of sites (S) that turned out to have relatively low affinity for both GTPγS and GDP; the additional parameters were justified by significant decreases in the sum of squares ($P < 0.0001$). Analyses in terms of eq 4 therefore exhibit the expected consistency with those in terms of eq 1; with the latter, the minimum value of n is 2 for binding in the presence of ATPγS and 3 for that under all other conditions.

Table II: Effects of Adenyl Nucleotides and Carbachol on the Binding of Guanyl Nucleotides in Terms of Scheme II[a]

ATPγS (mM)	AMP-PNP (mM)	carbachol (mM)	log K_{AR} (GDP)	cooperativity factors[b] log *a* (GTPγS/GTPγS)	log *b* (GDP/GDP)	log *c* (GTPγS/GDP)	capacity (nmol/g of protein) R_t	S_t	F'_3[c]
0.0	0.0	0.0	−7.81 ± 0.17	0.43 ± 0.23	1.72 ± 0.15	−0.03 ± 0.03	1.8 ± 0.3	99 ± 17	0.24
0.0	0.0	2.0			2.50 ± 0.16[d]	−0.14 ± 0.02[d]			
0.1	0.0	0.0	−7.15 ± 0.22[e]	1.18 ± 0.35[e]	0.86 ± 0.17[f]	−0.14 ± 0.04	3.3 ± 0.1[g]	0.0[h]	0.0[h]
0.1	0.0	2.0			1.04 ± 0.18[f]	−0.15 ± 0.03			
0.0	0.1	0.0	−6.40 ± 0.12[e]	*i*	0.68 ± 0.15[f]	−0.40 ± 0.02[f]	3.9 ± 0.8[g]	272 ± 55[g]	0.30
0.0	0.1	2.0			1.30 ± 0.14[d,f]	−0.51 ± 0.02[d,f]			

[a] Twenty-five sets of data acquired at graded concentrations of either GTPSγS or GDP were analyzed simultaneously according to eq 2, with $[P]_b$ computed according to Scheme II (eq 4). Measurements at graded concentrations of unlabeled GTPγS were performed in the absence (four experiments) and in the presence (three experiments) of ATPγS. Measurements at graded concentrations of GDP involved concomitant assays in the absence and presence of carbachol (2 mM); three experiments were performed under each set of conditions with respect to ATPγS and AMP-PNP (18 sets of data). Parameters were shared among multiple sets of data as follows: single values of log K_{PR}, log K_{PS}, and log K_{AS} for all data; single values of log K_{AR}, log *a*, and F_D for all data acquired both with and without carbachol under the same conditions with respect to adenyl nucleotide; single values of log *b* and log *c* for all data acquired under the same conditions with respect to carbachol and adenyl nucleotide; and separate values of $[R]_t + [S]_t$ and NS for the data from each experiment. The fitted values of log K_{AR}, *a*, *b*, and *c* are listed in the table. Fitted values of $[R]_t + [S]_t$ were normalized per gram of protein, and the appropriate value of F_D was used to calculate R_t and S_t for each experiment; individual values then were averaged to obtain the means (±SEM) listed in the table. The fitted values of F_D are 0.017 ± 0.004 and 0.014 ± 0.004 for binding in the absence of adenyl nucleotide and in the presence of AMP-PNP, respectively. The fitted values of parameters common to all of the data are as follows: log K_{PR} = −7.45 ± 0.04 (GTPγS), log K_{PS} = −5.48 ± 0.11 (GTPγS), and log K_{AS} = −3.22 ● 0.06 (GDP). The data are illustrated in Figures 1 and 4, and further details are described in the legend to Figure 4. [b] The relative affinity of the ligand for the vacant site of a half-occupied dimer and for a vacant dimer. [c] The fraction of observed specific binding attributable to S. Equation 1 was fitted to the simulated data illustrated in Figure 4, which in turn represent the best fit of eqs 2 and 4 as described in footnote *a* above. The parametric values are as follows; those listed for log K_2 and F'_2 are for binding in the absence and presence of carbachol, respectively: no adenyl nucleotide (n = 3) (Figure 4A), log K_1 = −8.08, log K_2 = −5.77 and −5.00, log K_3 = −3.22, F'_2 = 0.42 and 0.53, and F'_3 = 0.24; with ATPγS (n = 2) (Figure 4B), log K_1 = −7.38, log K_2 = −5.97 and −5.79, and F'_2 = 0.73 and 0.73; with AMP-PNP (n = 3) (Figure 4C), log K_1 = −6.70, log K_2 = −5.38 and −4.73, log K_3 = −3.21, F'_2 = 0.92 and 1.07, and F'_3 = 0.30. Depletion of [^{35}S]GTPγS leads to a small effect of carbachol on the values obtained for log K_1 (≤0.031 log unit), and the means are listed above; corresponding effects on log K_3 and F'_3 are negligible. In the absence of depletion, those parameters would be unchanged owing to the constraints applied in eq 4. [d] $P < 0.0001$ for the comparison with the corresponding value measured in the absence of carbachol (F statistic). [e] $P \leq 0.01$ for the comparison with the corresponding value measured in the absence of adenyl nucleotide (F statistic). [f] $P < 0.0001$ for the comparison with the corresponding value measured in the absence of adenyl nucleotide (F statistic). [g] $P < 0.01$ for the comparison with the corresponding value measured in the absence of adenyl nucleotide (t statistic). [h] F_D was fixed at 1 for all data acquired in the presence of ATPγS. [i] This parameter is essentially undefined by the present data, and the value proved to be indistinguishable from that determined either in the absence of adenyl nucleotide or in the presence of ATPγS; the values of other parameters are unaffected over that range.

To describe the binding of GDP and GTPγS in terms of Scheme II requires up to seven parameters, excluding the asymptotes: one microscopic dissociation constant for the binding of each ligand to either site of the vacant dimer (K_{PR}, K_{AR}), one dissociation constant per ligand for the sites identified as S (K_{PS}, K_{AS}), and three cooperativity factors (*a*, *b*, *c*). Since two ligands are involved, the latter can be either homotropic (*a*, GTPγS; *b*, GDP) or heterotropic (*c*, GTPγS and GDP). Eight sets of conditions are represented with respect to GDP, GTPγS, carbachol, ATPγS, and AMP-PNP; accordingly, there is a maximum of 48 such parameters if single values of each are common to the data from multiple experiments performed under the same conditions. In addition, each of the 25 sets of data potentially is associated with unique values of $[R]_t + [S]_t$, F_D, and NS for a maximum of 123 parameters. Some parameters are defined under some conditions but not others; for example, the affinity and homotropic cooperativity of GTPγS are well-defined by isotopic dilution, but neither can be estimated from the inhibitory behavior of GDP at a single concentration of the radioligand, at least in the absence of adenyl nucleotides. Other parameters may be redundant; for example, carbachol is without effect on either K_{PR} or K_{PS}. A series of analyses therefore was carried out in which the number of parameters was reduced in a stepwise manner to obtain the set listed in Table II; the fit is consistent with all of the data and defined by a unique minimum in the weighted sum of squares. The fitted curves are illustrated in Figure 4 for experiments that included GDP; the fit to data acquired by isotopic dilution is virtually superimposable on the fits obtained with the multisite model and illustrated in Figure 1B.

The result summarized in Table II was achieved without an appreciable increase in the sum of squares over that obtained from various analyses involving larger numbers of parameters ($P > 0.15$); further simplification generally was deleterious to the fit, as indicated in the footnotes to the table. All dissociation constants and cooperativity factors are defined independently, as indicated by the off-diagonal elements of the covariance matrix. The affinity of GTPγS for the vacant dimer (K_{PR}) could be taken as identical for all 25 sets of data; the corresponding affinity of GDP (K_{AR}) was unaffected by carbachol but sensitive to both ATPγS and AMP-PNP. The homotropic cooperativity factor for GTPγS similarly was unaffected by carbachol but sensitive to ATPγS, while the homotropic cooperativity factor for GDP and the heterotropic cooperativity factor generally were sensitive to both carbachol and adenyl nucleotides. The affinities of GTPγS and GDP for the sites identified as S could be held common to all of the data (K_{PS}, K_{AS}), and thus appear to be insensitive to either carbachol or AMP-PNP. Capacity was unaffected by carbachol but sensitive to adenyl nucleotides. Both ATPγS and AMP-PNP increased R_t by about 2-fold; S_t was reduced to zero in the presence of ATPγS and increased 2.7-fold by AMP-PNP.

Agonist-dependent effects arise exclusively from changes in the cooperative properties associated with GDP. In the absence of adenyl nucleotide and in the presence of AMP-PNP, negative homotropic cooperativity is increased 4–6-fold (*b*, $P < 0.0001$), and positive heterotropic cooperativity is either induced or increased (*c*, $P < 0.0001$). There is relatively little effect in the presence of ATPγS, as illustrated by the data presented in Figure 4B; a 1.5-fold increase in *b* is only marginally significant ($P = 0.05$), and *c* is unchanged ($P = 0.45$). The agonist is without effect on the affinity of either GTPγS or GDP for the vacant dimer or on the homotropic cooperativity of GTPγS. The lack of change in K_{PR}, *a*, K_{PS}, R_t, and S_t reflects the failure of carbachol to affect the binding of GTPγS in the absence of GDP.

Changes brought about by adenyl nucleotides are more complex than those associated with carbachol, and some cannot be accommodated within the context of Scheme II. Both ATPγS and AMP-PNP decrease the affinity of GDP for the unliganded dimer ($P < 0.008$), and adenyl nucleotides affect all three of the cooperativity factors: ATPγS increases the negative homotropic cooperativity of GTPγS ($P = 0.01$), both nucleotides decrease the negative homotropic cooperativity of GDP ($P < 0.0001$), and AMP-PNP increases positive heterotropic cooperativity ($P < 0.0001$). The latter effect of AMP-PNP and its enhancement by carbachol account for the GDP-dependent increase in the binding of the radioligand (Figure 4C). Both ATPγS and AMP-PNP increase R_t and affect S_t as described above. The adenyl nucleotide-dependent effects on affinity (K_{AR}) and cooperativity (a, b, and c) can be rationalized in terms of Scheme II, but those on capacity cannot. The apparent disappearance of S in the presence of ATPγS could reflect competition between ATPγS and the radioligand; in contrast, the AMP-PNP-dependent increase in S_t and the increases in R_t suggest that the model may be incomplete, at least with respect to the role of adenyl nucleotides.

DISCUSSION

Selectivity of Labeling and Identity of the Labeled Sites. Guanyl nucleotide-specific sites outnumber muscarinic receptors by at least 60-fold in cardiac membranes (Ehlert, 1985; Hilf et al., 1989; Birnbaumer et al., 1990), but muscarinic agonists cause a marked, GDP-dependent increase in the specific binding of [^{35}S]GTPγS. When membranes from hamster left ventricle were assayed in the absence of adenyl nucleotide, the carbachol-dependent contribution represented 40% of total specific binding at 160 pM [^{35}S]GTPγS and the optimal concentration of GDP; the increase was equivalent to 26% of specific binding in the absence of GDP. Similar results have been reported for sarcolemmal membranes from porcine atria, where the density of muscarinic receptors was about 10-fold higher than in crude preparations: at optimal concentrations of GDP, carbachol was found to affect up to 40% of the sites labeled in the presence of 0.3–5.0 nM [^{35}S]-GTPγS (Hilf et al., 1989). The agonist was proportionately less effective at higher concentrations of the radioligand, both in hamster left ventricle and in the atrial preparations studied previously (Hilf et al., 1989). Carbachol-sensitive sites thus appear to be labeled preferentially at low concentrations of [^{35}S]GTPγS, the relative scarcity of muscarinic receptors notwithstanding.

The agonist-dependent increase in specific binding is an empirical quantity estimated from the data per se; it is independent of mechanistic considerations. The value at the optimal concentration of GDP in the absence of adenyl nucleotide places a lower limit of 26% on the fraction of labeled sites linked to muscarinic receptors. An explicit value for the number of receptor-linked sites requires a mechanistic scheme for the binding of GDP and [^{35}S]GTPγS, since the effect of carbachol seems to be absolutely dependent upon GDP; no effect was observed with GTPγS alone or in the presence of GMP-PNP.[3] A resolution of this problem is suggested by the effects of adenyl nucleotides, which regulate the binding of guanyl nucleotides in an obscure but apparently cooperative manner. As described below, data acquired in the presence of AMP-PNP reveal that cooperativity also accounts at least in part for the interaction of GDP and GTPγS with the labeled sites. AMP-PNP thus resolves the mechanistic ambiguity otherwise inherent in the binding of guanyl nucleotides. The data are in excellent agreement with Scheme II, which comprises both a monovalent component and an agonist-sensitive, bivalent component that accounts for the cooperative effects.

With a plausible model in hand, the effect of GDP can be used to infer the exact number of labeled sites associated with muscarinic receptors. The value is given explicitly by eq 4 minus the term in PS, but a good estimate can be obtained by comparison with eq 1 ($n = 3$) (Table II). Binding attributable to the putative dimer in Scheme II corresponds to the "sites" ostensibly of high and intermediate affinity for GDP in eq 1, while the monomer is represented by the sites of low affinity. In the absence of an adenyl nucleotide and in the presence of AMP-PNP, the carbachol-sensitive dimer accounts for 70–76% of specific binding in the absence of GDP; the carbachol-insensitive monomer accounts for the balance (Table II). Agonist-sensitive sites appear to account for 100% of specific binding in the presence of ATPγS, since the dimer alone is sufficient to describe the data. Receptor-linked sites thus constitute most or all of the signal at 160 pM [^{35}S]GTPγS when the data are interpreted in the context of Scheme II.

The comparatively large contribution of the putative dimer reflects the selectivity of the radioligand as it emerges from the model. The fitted estimates of affinity indicate that the radioligand binds 93-fold more tightly to the vacant dimer ($\log K_{PR} = -7.45$) than to the monomer ($\log K_{PS} = -5.48$). In contrast, the estimates of R_t and S_t indicate that the dimer accounts for only 3.5% of total capacity in the absence of adenyl nucleotide and for only 2.9% in the presence of AMP-PNP [i.e., $2R_t/(2R_t + S_t)$]. A subpopulation of high-affinity, low-capacity sites is consistent with the notion of selective labeling, although the capacity is estimated by extrapolation; the values of R_t and S_t are linearly dependent upon the estimates of K_{PR} and K_{PS} at the low concentration of [^{35}S]-GTPγS used in the assays. Also, the dimer can account for the entire signal in the presence of ATPγS (i.e., $S_t = 0$).

Receptor-mediated effects on binding suggest that most or all of the labeled sites are G proteins. This view is supported by the striking similarity between the data illustrated in Figure 4A and the results of previous studies in which purified muscarinic receptors were reconstituted with either G_i (Tota et al., 1987) or G_o (Ikegaya et al., 1990). Also, the carbachol-stimulated binding of [^{35}S]GTPγS to sarcolemmal preparations from porcine atria was reduced by 70–80% upon pretreatment of the membranes with pertussis toxin and NAD^+ (Hilf et al., 1989). Since the effect of carbachol is associated with the bivalent component R in Scheme II, the molecular species responsible for the cooperative properties may be a dimer of holo-G proteins or G_α subunits. A heterodimer of G_o and G_i is suggested by the observation that muscarinic receptors from ventricular myocardium copurify with roughly equivalent amounts of $G_{o\alpha}$ and $G_{i\alpha}$, as identified by western blots with G_α-specific antibodies (Matesic et al., 1991).

The apparent selectivity of [^{35}S]GTPγS may reside in the intrinsic affinity of G proteins that interact with muscarinic receptors, or it may emerge as a consequence of that interaction. Cardiac muscarinic receptors appear to be exclusively M_2 in mammals, as determined by northern blots (Peralta et al., 1987; Maeda et al., 1988), immunospecificity (Luetje et al., 1987; Li et al., 1991), and the binding of subtype-specific ligands (Watson et al., 1986a,b; Deighton et al., 1990). They inhibit adenylate cyclase via G_i (Gilman, 1987), which exhibits higher affinity for guanyl nucleotides than does G_s (Bokoch et al., 1984). A preference of [^{35}S]GTPγS for G_i over G_s is consistent with our observation that isoproterenol is without effect on the binding of [^{35}S]GTPγS either with

or without GDP,[3] at least under the conditions of the experiments represented in Figure 4A. Higher affinity for GTPγS also may be related to the oligomeric form of the G protein [e.g., $(\alpha\beta\gamma)_2$] or to the presence of muscarinic receptors. The possibility that muscarinic receptors increase the affinity of G proteins for [^{35}S]GTPγS recalls the observation that G proteins increase the number of reconstituted M_2 receptors that exhibit higher affinity for muscarinic agonists (e.g., Tota et al., 1987; Florio & Sternweis, 1989; Ikegaya et al., 1990).

Sites Recognized by Adenyl Nucleotides. Most or all of the sites labeled by [^{35}S]GTPγS in the present investigation appear to be G proteins, but the sites mediating the effects of adenyl nucleotides remain unidentified. Increased binding in the presence of ATP, ATPγS, AMP-PNP, ADP, or ADPβS suggests that adenyl nucleotides and [^{35}S]GTPγS bind concomitantly under at least some conditions, but it remains unclear whether or not the effects are strictly allosteric; adenyl and guanyl nucleotides may recognize pharmacologically distinct sites, or binding may be cooperative but ultimately competitive as in the example of Scheme II. Potential targets specific for adenyl nucleotides include adenylate cyclase (Birnbaumer et al., 1990) and the P_2 purinergic receptors (Watson & Abbott, 1990), although the present results lend little support to either possibility. Alternatively, adenyl and guanyl nucleotides may recognize separate sites on the G protein, a possibility suggested by the observation that eucaryotic initiation factor 2 (eIF-2) binds adenyl nucleotides at a site distinct from that which binds guanyl nucleotides (Gonsky et al,, 1990).

Some evidence suggests that the selectivity of nucleotide-specific sites for a particular base is not absolute. Although ATP and AMP-PNP reportedly are without effect on the binding of [^{35}S]GTPγS to purified G_i (Bokoch et al., 1984), ATP has been found to be inhibitory at purified G_s reconstituted with β-adrenergic receptors (Asano et al., 1984). Also, ATPγS[3] as well as ATP and ADP (Rodbell et al., 1971; Birnbaumer & Pohl, 1973; Williams & Lefkowitz, 1977) can have GTP-like effects on the binding of agonists to G protein-linked receptors. Finally, ATP mimics GTP in enhancing the response of adenylate cyclase to stimulatory agents [summarized in Birnbaumer and Yang (1974)]. These considerations suggest that adenyl nucleotides can substitute for guanyl nucleotides under appropriate conditions and that the present observations may reflect the binding of ATP, ADP, AMP-PNP, and ATPγS to the GTP-specific site of G proteins.

Guanyl nucleotides generally are effective at concentrations lower than those required of adenyl nucleotides (e.g., Rodbell et al., 1971; Birnbaumer & Yang, 1974). In some studies, GTP-like effects of ATP and its analogues may have resulted from contamination of the adenyl nucleotide with the corresponding guanyl nucleotide (Kimura et al., 1976) or from an enzymic conversion of the former to the latter (Otero et al., 1988). Such possibilities are unlikely to account for the present results, since adenyl nucleotides and their guanyl counterparts exhibit different effects on the binding of [^{35}S]-GTPγS.

Whatever the identity of the sites recognized by adenyl nucleotides, it is of interest that myocytes contain millimolar concentrations of ATP and ADP (Robison et al., 1971; Barany et al., 1975; Burt et al., 1975). Intracellular levels of those compounds therefore are comparable to the concentrations that affect the binding of [^{35}S]GTPγS to membrane fragments. It follows that the noncompetitive effects observed in the present investigation may be relevant to the functioning of G proteins in vivo.

Cooperative Effects among Guanyl Nucleotides. The inhibitory behavior of GTPγS and GDP reveals a mechanistically ambiguous dispersion of affinities in the absence of an adenyl nucleotide and in the presence of ATPγS. For a system at thermodynamic equilibrium, at least three schemes could account for the data. First, two or more classes of distinct, noninterconverting, and mutually independent sites may differ in their affinity for the ligand (e.g., Scheme I). Second, intrinsically identical sites may exist in two or more interconverting states recognized by the ligand and arising from transient association with other, stoichiometrically limiting components of the membrane; if the labeled sites are G proteins and the transient complex involves muscarinic receptors, this possibility represents the reciprocal of the *mobile receptor* or *ternary complex* model for the binding of agonists in such systems (De Lean et al., 1980). Third, successive equivalents of the ligand may bind to interacting sites in a cooperative manner.

Whereas strictly inhibitory behavior is ambiguous, neither the first model nor the second can account for the dose-dependent *increase* in binding effected by GDP in the presence of AMP-PNP; rather, both schemes predict a reduction in the specific binding of [^{35}S]GTPγS at any concentration of GDP, irrespective of the values of the various parameters that define the system. With the mobile receptor model, this restriction applies not only to the common variant in which the populations of interacting G proteins and receptors are homogeneous (e.g., De Lean et al., 1980; Ehlert, 1985; Lee et al., 1986), but also to that extension in which distinct G proteins compete for the receptor (e.g., Minton & Sokolovsky, 1990; Leung et al., 1990).

The striking effect of GDP in the presence of AMP-PNP is readily interpreted in terms of cooperative schemes, which allow for simultaneous binding of GDP and the radioligand. If it is assumed that guanyl nucleotides bind according to the same mechanism under all conditions studied, the effect of AMP-PNP is to perturb the cooperative properties in a manner that eliminates the ambiguity inherent in data acquired either in the absence of adenyl nucleotide or in the presence of ATPγS. The assumption that binding is mechanistically consistent throughout is supported by the intermediate effect obtained with ATPγS: heterotropic cooperativity is increased over that in the absence of adenyl nucleotide, but the change is not sufficient to yield the bell-shaped pattern obtained in the presence of AMP-PNP. Also, Scheme II is in excellent agreement with all of the data, and the fit is achieved with relatively few parameters.

GDP and GTPγS each exhibit negative homotropic cooperativity under all conditions tested, while heterotropic cooperativity is negligible in the absence of adenyl nucleotide and carbachol. The agonist achieves essentially the same effects regardless of the situation with respect to adenyl nucleotides: there is an increase in the negative homotropic cooperativity of GDP, and positive heterotropic cooperativity is either induced or increased. The changes therefore can be rationalized in terms of only two parameters (i.e., b and c), superficial differences in the binding patterns notwithstanding. It is of interest that carbachol only affects parameters related to affinity; since capacity is unaffected, receptor-mediated events appear to be independent of the total concentration of G proteins. The selective effect of carbachol on cooperativity is entirely consistent with Scheme II, although neither the agonist nor the receptor appears explicitly in the model as formulated here. There appear to be two states of the homo- or heterooligomer represented by R; one state may occur only in the presence of agonist, or the agonist may perturb a preexisting equilibrium between the two. In either event, the

net effect of carbachol is to favor the binding of GTPγS over that of GDP at the second site of the putative dimer. Agonist-induced changes in cooperativity that facilitate the exchange of GTP for bound GDP may constitute the mechanism of G protein activation in vivo.

A further comment here concerns the suggestion that at least 70% of the labeled sites are G proteins linked to muscarinic receptors. That estimate is based upon Scheme II and, in particular, upon the assumption that R represents a homogeneous population of bivalent or dimeric forms. If the sites identified as R are in fact heterogeneous, some may not be under muscarinic control. In that event, the receptor-linked sites would account for less than 70% of specific binding in the absence of GDP. Commensurately larger changes in cooperativity then would be required in order that fewer sites could account for the carbachol-dependent effects illustrated in Figure 4.

Adenyl nucleotides resemble carbachol in that they affect cooperativity involving GDP; they also reduce the affinity of GDP for the vacant dimer and the homotropic cooperativity of GTPγS (Table II), effects that could be rationalized in the manner described above for carbachol. Unlike the agonist, however, AMP-PNP and ATPγS increase R_t and either increase S_t or reduce it to negligible levels. The apparent changes in capacity are difficult to rationalize in terms of Scheme II, owing in part to uncertainty over the sites recognized by adenyl nucleotides. A similar question does not arise with carbachol, which presumably acts in a strictly noncompetitive manner.

If adenyl and guanyl nucleotides compete for both sites of a dimer, changes in R_t could reflect the absence of ATPγS or AMP-PNP from the model as presently formulated: Scheme II allows for only two ligands, the probe and GDP [i.e., eqs 171–173 with $i = 2$ in Wells (1992); see also eq 158]. The effect of ATPγS on the binding of [^{35}S]GTPγS in the absence of GDP is in excellent agreement with Scheme II, and the fitted curve is virtually identical to that illustrated in Figure 2A. While a competitive effect may be a contributing factor, the problem cannot be resolved simply by increasing the number of independent variables in the present model. The effect of AMP-PNP in the absence of GDP implies that the cooperative oligomer contains at least three sites if binding is fully competitive (Figure 2B), and at least four sites are implied by the effects of ADP and ATP (Figure 2C,D). Also, both ATPγS and AMP-PNP affect the capacity of the carbachol-insensitive sites identified as S in Scheme II. Concomitant effects on R_t and S_t suggest that it may be inappropriate to assume that R and S are unrelated, notwithstanding the consistent pattern observed with carbachol; some or all of the sites identified as S may interact with R, further suggesting that the relevant oligomer contains more than two binding sites for guanyl nucleotides. This notion is supported by the observation of Vaillancourt et al. (1990) that up to three α subunits of transducin can be cross-linked by means of pertussis toxin together with a radiolabeled, photoactivatable derivative of NAD^+.

Previous observations from several laboratories are consistent with the suggestion that G protein dimers or larger oligomers bind guanyl nucleotides in a cooperative manner. Hill coefficients greater than 1 have been reported for the GTP-dependent activation of potassium channels linked to muscarinic receptors (Kurachi et al., 1990) and for the activation of transducin by rhodopsin (Wessling-Resnick & Johnson, 1987). Various studies have shown that the agonist-promoted release of radioligands from the GTP-specific site of G proteins is facilitated by unlabeled nucleotides (Cassel & Selinger, 1978; Fung & Stryer, 1980; Michel & Lefkowitz, 1982; Murayama & Ui, 1984). Although the latter may serve in part to block reassociation of the radioligand, the obligatory nature of their contribution in some studies (e.g., Michel & Lefkowitz, 1982; Murayama & Ui, 1984) suggests a cooperative effect wherein the rate constant for dissociation from one site of an oligomer is increased by the occupancy of another. Cooperative effects also may account for the biphasic dissociation of guanyl nucleotides from solubilized G_i (Bokoch et al., 1984; Carty et al., 1990) and reconstituted G_s (Brandt & Ross, 1985). Similarly, an oligomeric form of the G protein may account for the finding that β-adrenergic receptors enhance the exchange of GDP at approximately 50% of the molecules of G_s in reconstituted systems, irrespective of the concentration of receptors (Rubenstein et al., 1991).

Implications for the Binding of Agonists. The dispersion of affinities revealed by GDP at the sites labeled by [^{35}S]-GTPγS recalls that revealed by agonists at cardiac muscarinic receptors. At least three forms or states can be identified when the data are described as a sum of hyperbolic terms (eq 1), both for GDP with and without carbachol (Figure 4) and for agonists with and without guanyl nucleotides [Chidiac et al. (1991); Wong et al. (1986) and references cited therein]. Also, agonist-induced changes in the binding of GDP mimic the characteristic effect of guanyl nucleotides on the binding of agonists; that is, the allosteric ligand promotes an apparent interconversion of G proteins or receptors from higher to lower affinity for GDP on the one hand and for agonists on the other. The reciprocal nature of these interactions points to a common underlying mechanism, a view that is supported by the observation that similar results can be obtained with purified receptors and G proteins in reconstituted preparations (Tota et al., 1987).

The binding patterns typically revealed by agonists and GDP at their respective sites also are similar in their ambiguity, at least in the absence of AMP-PNP. Such data commonly are analyzed in terms of distinct and independent sites (i.e., Scheme I), an essentially empirical formulation that is silent on the factors governing the distribution of G proteins or receptors among the different states. For binding to the receptor, mechanistic interpretations typically involve one or another variant of the mobile receptor hypothesis: that is, a system in which the dispersion of affinities reflects an agonist- and nucleotide-regulated equilibrium between free receptor and a receptor–G protein complex (Birnbaumer et al., 1990). Such schemes are qualitatively attractive and complement the view that G protein-mediated transduction involves a transient complex between the G protein and the receptor. When examined quantitatively, however, the mobile receptor model is difficult to reconcile with the binding of agonists and, in particular, with the effects of guanyl nucleotides (Lee et al., 1986; Ehlert & Rathbun, 1990).

The problem seems to lie in the notion of a random process wherein all receptors are equally accessible to all G proteins on the time scale of a binding assay. Any scheme in which the receptor and G protein exchange rapidly between free and coupled forms leads to the unfulfilled prediction that the binding of agonists will be sensitive to the effective concentration of each macromolecule: that is, the local concentration relative to the constants that govern the degree of coupling. Muscarinic receptors in preparations of solubilized myocardium exhibit the dispersion of affinities and the sensitivity to guanyl nucleotides characteristic of those in native membranes (e.g., Berrie et al., 1984; Poyner et al., 1989), despite the likelihood that solubilization alters the local concentrations of receptors and G proteins. Also, the effects of GMP-PNP

and magnesium on solubilized histaminergic receptors are reversible, in that the sites can be made to interconvert from one state to the other and back (Wells & Cybulsky, 1990). Finally, purified muscarinic receptors from brain and heart appear heterogeneous to agonists when reconstituted with a 5–200-fold molar excess of G_o or G_i (Tota et al., 1987; Florio & Sternweis, 1989; Ikegaya et al., 1990); in contrast, exchange-based models predict that the sites will appear homogeneous at molar ratios of G protein to receptor of about 2:1 or more (Lee et al., 1986).

It has been pointed out from time to time that the characteristic binding patterns exhibited by agonists at G protein-linked receptors might arise from cooperative interactions within an oligomeric array (e.g., Limbird et al., 1975; Birdsall et al., 1978; Sokolovsky et al., 1983; Mattera et al., 1985). This possibility is favored by the present evidence that cooperative effects are at least partly responsible for the binding patterns described by GDP and by the likelihood that agonists and GDP bind according to a common mechanism. Cooperativity implies that the multiple states discerned by agonists are an intrinsic property of the receptor, a corollary that is supported by data on purified constituents reconstituted in phospholipid vesicles. The binding of agonists to purified muscarinic receptors from heart is multiphasic even in the absence of added G protein, which promotes but apparently is not essential for the high-affinity form (e.g., Peterson et al., 1984; Ikegaya et al., 1990). Also, Hill coefficients of 1.4–1.5 have been reported for the binding of [^{3}H]quinuclidinylbenzilate to cardiac muscarinic receptors in sarcolemmal (Mattera et al., 1985) and plasma membranes (Boyer et al., 1986). Finally, studies on the photoaffinity labeling of muscarinic receptors (Avissar et al., 1983), the radiation inactivation of α- and β-adrenergic receptors (Venter & Fraser, 1983), the cross-linking of glucagon receptors (Herberg et al., 1984), and the hydrodynamic properties of cardiac muscarinic receptors (Peterson et al., 1986) all suggest that G protein-linked receptors occur in pairs or larger aggregates. The possibility that M_2 muscarinic receptors occur in pairs has been suggested previously (Potter et al., 1991), and a cooperative oligomer might account for the noncompetitive effects observed with gallamine (Stockton et al., 1983).

The Hill coefficient of 1.4 obtained for [^{3}H]quinuclidinylbenzilate in the presence of GMP-PNP prompted Mattera et al. (1985) to analyze their data in terms of a bivalent receptor, presumably a dimer. The effects of GMP-PNP on the binding of carbachol and other agonists emerged partly as changes in the cooperative interactions between the agonist and the radiolabeled probe. Agonists revealed three states of affinity, however, which appears to be a characteristic of M_2 receptors under appropriate conditions; the fitted model therefore included a noncooperative form analogous to S in Scheme II. Somewhat awkwardly, such an arrangement implied that guanyl nucleotides also promote an interconversion of some but not all of the receptors from the cooperative to the noncooperative form. If the three states of affinity recognized by GDP at the sites labeled by [^{35}S]GTPγS and by agonists at muscarinic receptors are manifestations of the same phenomenon, the curious interconversion from one form of the receptor to another may reflect the limitations of a bivalent model; it follows that the functional oligomer may include more than 2 equiv of the receptor.

Implications for the Interaction between G Proteins and Receptors. If G proteins and receptors both function as oligomers, it follows that heterooligomers containing two or more equivalents of each may constitute the basic unit of transduction. Such a possibility recasts the question of the mechanism whereby receptors interact with G proteins to mediate the allosteric relationship between agonists and guanyl nucleotides. Objections to the mobile receptor model are based in part on the problems encountered when attempting to rationalize multiple states of affinity exclusively in terms of an equilibrium between free receptors and G proteins on the one hand and a 1:1 complex on the other (Lee et al., 1986). Those problems are avoided if the apparent heterogeneity is a consequence of cooperative interactions intrinsic to either the G protein or the receptor alone. If there is indeed an exchange of elements through a transient, heteromeric complex, the uncoupled forms are likely to be oligomers of receptor on the one hand and G proteins on the other (e.g., $R_2 + G_2 \rightleftharpoons R_2G_2$).

The notion of cooperativity avoids some of the problems otherwise associated with exchange-based models; in predicting multiple states of affinity, however, it also obviates the need for coexisting and rapidly exchanging populations of free and G-coupled receptors. A nondissociating heterooligomer can account for many of the properties typically attributed to a transient complex, and it therefore constitutes an alternative to prevailing schemes for the allosteric effects between guanyl nucleotides and agonists. Partially purified receptors have been found to retain their sensitivity to guanyl nucleotides, and agonists tend to increase the degree of receptor–G protein copurification (e.g., Limbird et al., 1980; Senogles et al., 1987); moreover, G proteins do not necessarily dissociate from the receptor in the presence of guanyl nucleotides (Matesic et al., 1989; Poyner et al., 1989). The stabilizing effect of agonists has been rationalized as an agonist-promoted coupling of two proteins that otherwise are uncoupled (e.g., Limbird et al., 1980). An alternative possibility is that conditions favoring copurification increase the likelihood that receptor–G protein heterooligomers retain their native structure upon removal from the membrane. Radiation inactivation of GTP-sensitive glucagon binding has revealed a functional molecular size of 670 kDa, suggesting that the receptor and its G protein exist in the plasma membrane as large multimeric complexes (Schlegel et al., 1979).

ACKNOWLEDGMENT

We are grateful to Mrs. Ria Dekker for her contribution to the binding assays and to Mrs. Roselie Damian for secretarial assistance.

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Registry No. GTPγS, 37589-80-3; ATPγS, 35094-46-3; AMP-PNP, 25612-73-1; ADP, 58-64-0; ATP, 56-65-5; ADPβS, 35094-45-2; GDP, 146-91-8; ITP, 132-06-9; UDP, 58-98-0; adenosine, 58-61-7; carbachol, 51-83-2.