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Compartmentation of Receptors and Guanine Nucleotide-Binding Proteins in NG108–15 Cells: Lack of Cross-talk in Agonist Binding among the α_2-Adrenergic, Muscarinic, and Opiate Receptors

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SUMMARY

Many different types of receptors couple to the inhibitory guanine nucleotide-binding protein (G protein) G_i. In NG108–15 neuroblastoma-glioma cells, α_{2b}-adrenergic, m4 muscarinic, and δ-opiate receptors all use G_i as a transducer. According to the ternary complex model of receptor-G protein interactions, agonists bind to these receptors with high affinity only in their G protein-associated form. Conversely, G protein affinity for the receptor is increased by agonist binding. We have developed an extended ternary complex model in which multiple receptors couple to a single G protein and we have examined two consequences of the model theoretically and experimentally. First, the simple ternary complex model can account for the observed high and low affinity agonist binding only when G protein is limiting; however, measurements show a significant excess of G protein over receptor. Could this paradox be explained by other receptors competing for the same G protein and limiting the amount of free G protein so that high and low affinity agonist binding would be seen? Our theoretical simulations show that this does not occur unless the receptors and G protein are present in a precise stoichiometric ratio and have an extremely high affinity, such as when agonists for both receptors are present. The second prediction of this model is that binding of an agonist at one receptor should produce competition for G protein used by another receptor. If the G protein pool were limiting and freely mobile, this would result in an unlabeled agonist at one receptor decreasing binding of a radiolabeled agonist to another receptor. Experimentally, the G protein was made limiting by a partial pertussis toxin treatment. Radioligand binding to α_{2b}-adrenergic and m4 muscarinic receptors in these pertussis toxin-treated NG108–15 membranes showed no cross-talk with the δ-opiate or muscarinic receptors, which are present in excess. This could occur because the different receptors interact with structurally different G proteins (e.g., distinct β or γ subunits). More likely it is because of limitations of the mobility of G proteins in the membrane due to 1) attachment to structural elements, such as the cytoskeleton, 2) sequestration in lipid pools, or 3) organization into slowly exchanging supramolecular complexes. These results show that we must reexamine the assumptions of the collision coupling and ternary complex models.

G protein-coupled transduction mechanisms are important in many different types of intracellular signaling systems (1–5). The mechanism of activation of G proteins by guanine nucleotides has been examined extensively in solubilized preparations (6) and receptor-mediated activation has been examined in reconstituted systems (7–10). The interactions of G protein with receptors in intact cells are less well understood.

Jacobs and Cuatrecasas (11) proposed the "mobile receptor" theory, which describes ligand binding to receptors that change affinity upon interactions with another membrane component. De Lean and co-workers (12, 13) coined the term "ternary complex model," which has been used extensively in the study of G protein-coupled receptors. According to this model, there are two affinity states of receptor for agonist, low affinity with receptor alone and high affinity with G protein bound to receptor.[1] Although the model includes these two affinity states, it cannot fully account for the two different binding affinities seen in equilibrium agonist binding assays (14). The TCM predicts that distinct high and low binding affinities are observed only when the amount of G protein is stoichiometric

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[1] Actually, the observed affinity is intermediate between the low affinity for receptor alone and the high affinity for receptor coupled to G protein. In general though, the observed affinity is closer to the high than the low affinity (14).

ABBREVIATIONS: G protein, guanine nucleotide-binding protein; G_i, inhibitory guanine nucleotide-binding protein; G_{i2}, subtype of inhibitory guanine nucleotide-binding protein; PIC, *para*-iodoclonidine; oxo M, oxotremorine M; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TCM, ternary complex model.

with that of receptor (12–14), but the physiological concentration of G protein has been shown to be 20–700-fold greater than the concentration of receptor (14–16). Under these conditions the TCM predicts that all agonist binding would be high affinity.

Another limitation of the model is that it includes only one type of receptor and G protein. Recent evidence suggests that receptor-G protein interactions are promiscuous; that is, one receptor can interact with multiple G proteins or multiple receptors can interact with the same G protein. Functional measurements of receptor-G protein coupling such as agonist-stimulated GTPase activity (17, 18) and inhibition of adenylyl cyclase (19–21) support the existence of promiscuous interactions. Given that cells often contain multiple receptors that are capable of interacting with the same G protein in reconstitution systems, the effective excess of G protein may be less than that expected from a simple molar ratio of G protein to a single receptor. No theoretical studies have been performed to examine the effects of a second receptor or a second G protein on equilibrium binding predicted by the TCM.

The presence of two receptors that both couple to the same G protein could also result in heterologous binding effects (or binding cross-talk). This would occur if both receptors coupled to a single pool of G protein and the amount of G protein was limiting (i.e., less than the sum of the two receptors). In this case, binding of an agonist to one receptor would cause the formation of a ternary complex, which would reduce the availability of the G protein to the second receptor. NG108–15 neuroblastoma × glioma cells are useful for examining this process because they have muscarinic, opiate, and α_2-adrenergic receptors, all linked to G_i (20–23).[2] Cross-talk among these three receptors in adenylyl cyclase inhibition assays has been demonstrated experimentally in NG108–15 cells (24), but there are no reports on cross-talk at the level of binding.

The aims of this paper were 1) to examine the predictions, with respect to agonist binding, of an extended TCM with multiple receptors and G proteins and 2) to test for cross-talk in the binding of agonists to distinct receptors coupled to G_i. The absence of cross-talk in the binding of these three G_i-coupled receptors, even when the G protein is made limiting by pertussis toxin treatment, suggests that α_2-adrenergic, m4 muscarinic, and δ-opiate receptors interact with distinct pools of G_i in NG108–15 cell membranes.

Experimental Procedures

Materials. [^{125}I]PIC (2200 Ci/mmol), [^{3}H]oxo M (90 Ci/mmol), and [^{3}H]yohimbine (90 Ci/mmol) were obtained from New England Nuclear (Boston, MA). [D-Pen^{2,5}]Enkephalin, Gpp(NH)p, and yohimbine were obtained from Sigma. Naltrexone was a gift of Dr. Fedor Medzihradsky (Ann Arbor, MI). Oxo M was obtained from Research Biochemicals Inc. UK-14,304 was a gift from Pfizer. Pertussis toxin was a gift from Dr. Nadine Cohen (Massachusetts Public Health Biologic Lab, Jamaica Plain, MA). All other chemicals were reagent grade or better.

NG108–15 membranes. NG108–15 cells were a gift from Dr. Marshall Nirenberg (National Institutes of Health, Bethesda, MD) and were grown as described (23). Cells from passages 17 through 40 were used. Once the cells were confluent, they were incubated in serum-free medium for 16–24 hr and treated with either 10 ng/ml pertussis toxin or vehicle for 10–16 hr, a length of time sufficient to reduce agonist binding to ~25% of control. Membranes were prepared as described previously (25). Briefly, NG108–15 cells were harvested and resuspended in hypotonic buffer (5 mM Tris·HCl, 5 mM $MgCl_2$, pH 7.5). Cells were disrupted with 10 strokes of a motor-driven Teflon-glass homogenizer (600 rpm) at 4°. Undisrupted cells and nuclear material were pelleted by centrifugation at 1000 × *g* for 5 min. The supernatant was collected and the pellet was homogenized and centrifuged a second time. Both supernatants were combined and the membranes were collected by centrifugation at 100,000 × *g* for 45 min. Membranes were stored at −70°; they were thawed and washed once before use by resuspension of the membranes in TME (50 mM Tris·HCl, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.6), followed by centrifugation for 45 min at 100,000 × *g*.

Equilibrium binding studies. Two radioligands were used for equilibrium binding studies, [^{125}I]PIC and [^{3}H]oxo M. Similar assay conditions were used for both radioligands. Membranes were incubated at room temperature with radioligand for 45 min (PIC) or 90 min (oxo M). Nonspecific binding was determined in the presence of 10 μM yohimbine for [^{125}I]PIC or 10 μM atropine for [^{3}H]oxo M. To test for cross-talk, unlabeled α_2-adrenergic (UK-14,304), muscarinic (oxo M), or opiate ([D-Pen^{2,5}]enkephalin) agonists were added to the binding assay at the indicated concentrations. Gpp(NH)p-sensitive binding was defined as the difference between binding in the presence and absence of 50 μM Gpp(NH)p. The incubations were quenched with the addition of 5 ml of ice-cold TM buffer (50 mM Tris·HCl, 10 mM $MgCl_2$, pH 7.6), filtered on Whatman GF/C filters, and washed three times with 10 ml of ice-cold TM buffer, using a Brandel filter apparatus. The amount of radioactivity in the samples was measured in a Beckman scintillation counter, in plastic vials containing 4.5 ml of Scintiverse scintillation fluid. Protein was determined according to the method of Lowry *et al.* (26).

Computer modeling of equilibrium binding. All simulations were calculated using one of three models, 1) the simple TCM, 2) a modification of the TCM that includes two receptors and one G protein (2R1G) (Fig. 1), and 3) a modification that includes two G proteins (1R2G) (see Appendix B for details). In the 2R1G model, the two receptors, R_1 and R_2, bind their respective agonists, D_1 and D_2, and couple to a single pool of G protein, G. Each receptor follows the standard TCM in which binding of agonist to the receptor alone is of low affinity (K_1 and K_{1b}) and binding to the RG complex is of high affinity (K_3 and K_{3b}). There is no direct interaction between R_1 and R_2 but they can indirectly affect each other by their mutual interaction with G. R_1 binding tightly to G as the result of formation of a D_1R_1G ternary complex would decrease the free pool of G that would be available to R_2. It is assumed that R_1, R_2, and G are all in one freely accessible pool. See Appendix A for the equations governing this model.

Theoretical simulations were performed using a program XTALK written in QuickBasic (Microsoft), which used Newton's method of estimating polynomial roots for solution of the cubic roots in the equilibrium equations. For these simulations, the affinity constants (Table 1) were similar to those reported by Thomsen *et al.* (27) for human platelet α_2-adrenergic receptors.[3] With these parameters, there is a small degree (~1%) of precoupling of R_1 and G and the ratio of the high and low affinity binding constants is 10^4. The ratio of G protein to receptor was 100, except where otherwise noted. This ratio is similar to that obtained experimentally in S49 lymphoma cell membranes, where the amount of G_s was 200 times that of the β-adrenergic receptor (15), and in human platelet membranes, where the concentration of pertussis toxin substrate, G_i, was 20–100-fold higher than that of the α_2-adrenergic receptor (14). For simulations of agonist binding to receptors in NG108–15 membranes, receptor concentrations were equal to those found in NG108–15 plasma membrane preparations, as meas-

[2] In undifferentiated NG108–15 cells, both α_2 and opiate receptors couple largely to G_{i2} (19, 21).

[3] Similar simulations were also done in which there was a greater precoupling of R and G (i.e., K_4 and K_{4b} equal to 10^{-9} M) or a smaller difference between high and low affinity for agonists (K_1/K_3 equal to 10^2). The results were similar and no conclusions were affected.

TABLE 1

Values for constants used in modeling

Constants are taken from the 2RIG model (see Fig. 1). Unless otherwise noted in figure legends, values listed were used in all calculations. See Appendix A for derivation of equations.

Constants	Values
	M
R_1	10^{-11}
R_2	(number shown) × 10^{-11}
G[a]	10^{-9}
K_1, K_{1b}	10^{-7}
K_2, K_{2b}	10^{-7}
K_3, K_{3b}	10^{-11}
K_4, K_{4b}	10^{-11}
α_2-Adrenergic	10^{-11}
Muscarinic	1.7×10^{-11}
δ-Opiate	3.6×10^{-11}

[a] For experiments involving the α_2-adrenergic, muscarinic, and δ-opiate receptors, G concentration was 5×10^{-10}.

ured by equilibrium radioligand binding assays (184 fmol/mg for α_2-adrenergic, 260 fmol/mg for muscarinic, and 500 fmol/mg for δ-opiate receptors). The G protein concentration used was 9 pmol/mg (or 50 times that of the α_2-adrenergic receptor).

For all theoretical simulations, the agonist was assumed to be in excess over receptor and was assumed to be able to interact freely with receptor. The movement of receptor and G protein through the membrane was assumed to be unconstrained. The entire pool of receptor could interact with the entire pool of G protein.

Agonist binding to receptor was simulated for equilibrium conditions, in the absence of GTP. To obtain K_d values and to prepare smooth curves through the simulated data, the data were fit with unweighted, nonlinear, least-squares routines in InPlot version 3.0 (GraphPad Software, San Diego, CA), using either a two-independent site fit or a one-site fit.

Results

Theoretical effect of multiple receptors on ligand binding. Fig. 1 shows a diagram of the 2R1G model in which the TCM is expanded to include two receptors and one G protein. Simulations were done to determine the effect of adding a second receptor (R_2) on agonist binding to the first receptor (R_1) (Fig. 2). Both receptors, R_1 and R_2, bind to the G protein with the same affinity and the concentration of G protein was fixed at 100 times the concentration of R_1. The concentration of R_2 was varied, and the binding of D_1 to R_1 was calculated. If no D_2 (drug binding to the second receptor) is present, R_2 has only a modest effect on D_1 binding to R_1 (Fig. 2A). This occurs only at high concentrations of R_2, i.e., a 20-fold excess over G protein (R_2 = 2000). Clearly, at least one receptor species must be in excess of G protein to significantly reduce the pool of available G protein.

The addition of an agonist (D_2) that binds to R_2 results in a large effect on D_1 binding (Fig. 2B). This effect is seen only if R_2 is present at concentrations equal to or greater than the concentration of G protein. A much smaller effect is seen if the concentration of R_2 is less than the concentration of G protein (R_2 = 0–95), even when D_2 is present. Increasing the concentration of R_2 beyond the concentration of G protein (R_2 = 105–2000) produces continued decreases in D_1 binding. This shows that the effect of R_2 is dependent on the R_2/G protein ratio, even when D_2 is present.

One of the questions that we wished to address with these simulations was whether the addition of the second receptor would result in nonhyperbolic equilibrium agonist binding.

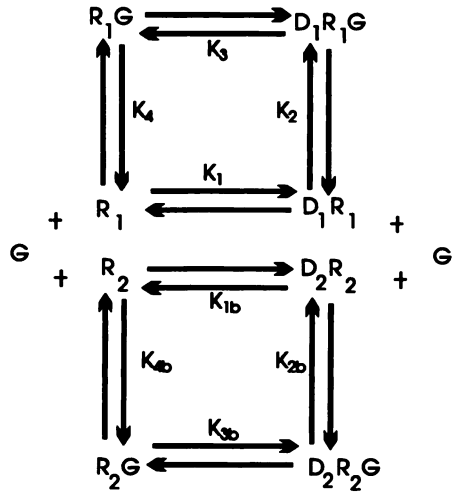

Fig. 1. Two receptors coupling to one G protein. An extension of the TCM to two receptors and one G protein (2R1G model) is illustrated. R_1 and R_2 are the two different receptors that interact with one G protein (G). D_1 and D_2 are agonists at the respective receptors. K_1 and K_{1b} are the low affinity equilibrium dissociation constants for D_1 and D_2 binding to the appropriate free receptor and K_3 and K_{3b} are the high affinity equilibrium dissociation constants for drugs binding to the RG complexes. K_4 and K_{4b} are the equilibrium constants governing the interaction of R_1 and R_2 binding, respectively, with the G protein in the absence of ligand. See Appendix A for specific definitions of the constants and solutions of the equations.

That is, can high and low affinity agonist binding to R_1 be produced in the TCM simply by adding a second receptor, R_2? Fig. 3 shows that adding a second receptor either alone or with D_2 does not generally cause D_1 to bind R_1 with two affinities. D_1 binds to R_1 with two apparent affinities only if the concentration of the second receptor is equal to that of G protein (R_2 = 100) and D_2 is present. If the concentration of R_2 is only slightly less (R_2 = 95) or slightly greater (R_2 = 105) than that of G protein, D_1 binds to R_1 with only one apparent affinity. Clearly, both the concentration requirements of R_2 and the dependence of this effect on the presence of D_2 indicate that simply adding a second receptor does not permit the TCM to adequately predict nonhyperbolic agonist-receptor interactions.

Because the model did show a decrease in D_1 binding to R_1 when both a second ligand and a second receptor were present, we examined the effect of D_2 concentration (Fig. 4). As described above, no change in D_1 binding was apparent when the concentration of the second receptor was less than that of G protein (R_2 = 50), even at high concentrations of D_2. When the concentration of R_2 was greater than that of G protein (R_2 = 200), increasing the concentration of D_2 rapidly decreased D_1 binding. The concentration of D_2 that produces a half-maximal decrease in affinity of D_1 is 10^{-9} M, a value between K_{1b} and K_{3b}. Increasing the affinity of R_2 for G protein and/or decreasing the affinity of R_1 for G protein by a factor of up to 100 does not qualitatively alter these results (data not shown).

Binding cross-talk between ligands in NG108–15 membranes. The 2R1G model predicts that adding an agonist for a second receptor will decrease agonist binding to the first receptor. NG108–15 cells provide an excellent system to test this prediction of the model because they have muscarinic, opiate, and α_2-adrenergic receptors, all of which couple to G_i. The α_2 and opiate receptors have been show to couple to the same subtype, G_{i2}. If this prediction of the model is correct, then binding of the α_2-adrenergic agonist [^{125}I]PIC to the α_2-

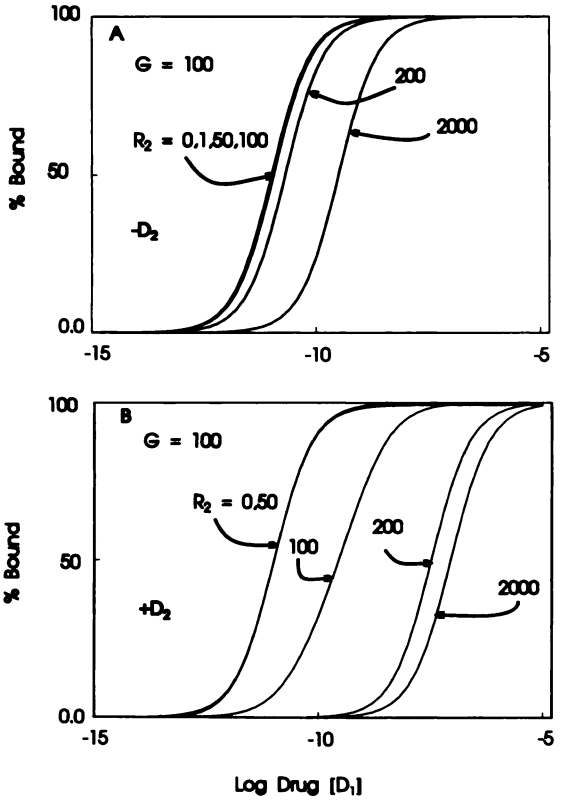

Fig. 2. Effect of two receptor types coupling to the same G protein. In both panels, the *solid lines* were derived from computer-generated data using the 2R1G model (see Fig. 1). The concentration of R_1 (first receptor) and G (G protein) were constant, and the concentration of R_2 (second receptor) was varied. The concentrations of R_2 and G indicated on this and subsequent figures are expressed relative to the concentrations of R_1, which is 10^{-11} M. Thus, G = 100 × R_1 or 10^{-9} M. In A, D_2 (agonist binding to the second receptor) is absent; in B, it is present. Theoretical data were prepared as described in Experimental Procedures. Sigmoidal concentration-binding curves were calculated from the theoretical data as described in Experimental Procedures. K_4 and K_{4b}, the dissociation constants for receptor-G protein interaction without ligand, were 10^{-11} M. For B, the concentration of D_2 was 10^{-7} M. The K_d values of both ligands for their respective receptors alone were 10^{-7} M and for receptor coupled to G protein were 10^{-11} M. A, Each *curve* represents binding of D_1 to R_1 at a different concentration of R_2, in the absence of D_2. Note that increasing the concentration of R_2 caused a rightward shift in agonist binding to R_1. B, Each *curve* represents binding of D_1 to R_1 at a different concentration of R_2, in the presence of a constant concentration of D_2 (10^{-7} M).

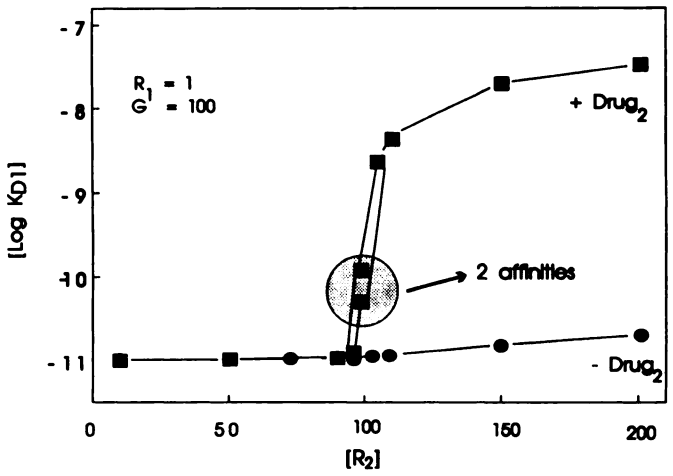

Fig. 3. Effect of R_2 concentration with (■) or without (●) D_2 on the affinity of agonist for R_1. Dissociation constants for D_1 binding to R_1 were calculated from the theoretical data in Fig. 2 and related simulations. Each *data point* represents the affinity of D_1 for R_1 at a given concentration of R_2, with higher affinities being higher on the graph. One *double point* is observed, representing nonhyperbolic binding of D_1 to R_1. This occurs when D_2 is present and the concentration of R_2 is 10^{-9} M (*shaded circle*).

adrenergic receptor should be reduced in the presence of an agonist for either the muscarinic or the opiate receptor, both of which outnumber the α_2-adrenergic receptor by roughly 2:1 or 3:1, respectively. Agonist binding to the muscarinic receptor should also be reduced in the presence of opiate agonists. The effects of α_2-adrenergic and muscarinic agonists on binding to the opiate receptor would be smaller, because the opiate receptor outnumbers both of the other receptors.

The theoretical predictions of the effect of the opiate agonist [D-Pen^{2,5}]enkephalin and the muscarinic agonist oxo M on binding of the α_2-adrenergic agonist [^{125}I]PIC are shown in Fig. 5A. In control membranes, the 2R1G model predicts that neither agonist should alter [^{125}I]PIC binding (Fig. 5A). This is true because the concentration of G protein is greater than the sum of the concentrations of all three receptors. Simulated pertussis toxin treatment, in which the G protein level becomes limiting, reduces [^{125}I]PIC binding. Under these conditions, both opiate and muscarinic agonists should cause a sharp decrease in [^{125}I]PIC binding. The effect is fully saturated at agonist concentrations of 10^{-6} M and does not increase further with increasing agonist. The model thus predicts that cross-talk should cause markedly decreased binding to the α_2-adrenergic receptor, as long as the concentration of G protein is limiting. Any significant reduction in agonist binding by pertussis toxin indicates that G protein concentration available to the α_2-adrenergic receptor is limiting, so the results are not dependent on precisely determining the amount by which pertussis toxin treatment reduces active G protein.

The effects of addition of either [D-Pen^{2,5}]enkephalin or oxo M on [^{125}I]PIC binding are shown in Fig. 5B. For membranes not treated with pertussis toxin, the theoretical and the experimental results agree, because no effect of either agonist on [^{125}I]PIC binding is seen. However, the 2R1G model predicted that after pertussis toxin treatment both the muscarinic and the δ-opiate agonist should markedly decrease PIC binding. In contrast, the experimental results show that neither agonist was able to reduce [^{125}I]PIC binding, even after pertussis toxin treatment. This directly contradicts the predictions of the model. Clearly, neither the TCM nor the 2R1G model is able to adequately predict receptor-G protein interactions, as examined by agonist binding.

Although the theoretical predictions do not match experimentally observed results for [^{125}I]PIC binding to the α_2-adrenergic receptor, it is possible that this is peculiar to the α_2-adrenergic receptor. The other two receptors coupled to G_i in NG108–15 cells may actually exhibit cross-talk. To examine this possibility, we performed a similar binding experiment to assess the effect of [D-Pen^{2,5}]enkephalin on [^{3}H]oxo M binding to muscarinic receptors in NG108–15 membranes. According to the 2R1G model with limiting G protein, [D-Pen^{2,5}]enkephalin binding to opiate receptors should reduce [^{3}H]oxo M binding to the muscarinic receptor. Again, the experimental results do not agree with the theoretical predictions (Fig. 6). [^{3}H]Oxo M binding is reduced somewhat in membranes from both control cells and pertussis toxin-treated cells in the presence

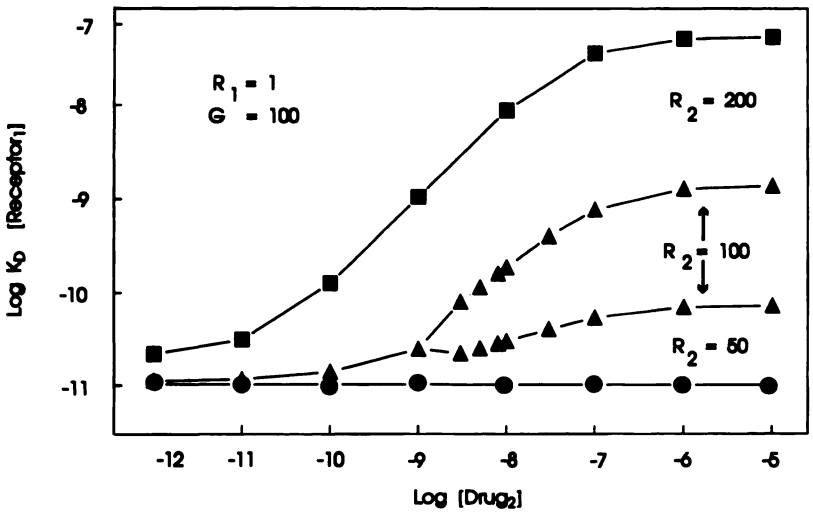

Fig. 4. Effect of D_2 concentration on D_1 binding to R_1. The affinity of D_1 for R_1 was calculated at the indicated concentrations of R_2 and D_2, as described in Fig. 3. *Double points* represent nonhyperbolic ligand binding. The concentration of R_2 was either 5×10^{-10} M (●), 10^{-9} M (▲), or 2×10^{-9} M (■). Note that only when the concentration of R_2 is equal to that of G protein (10^{-9} M) is nonhyperbolic agonist binding to R_1 observed.

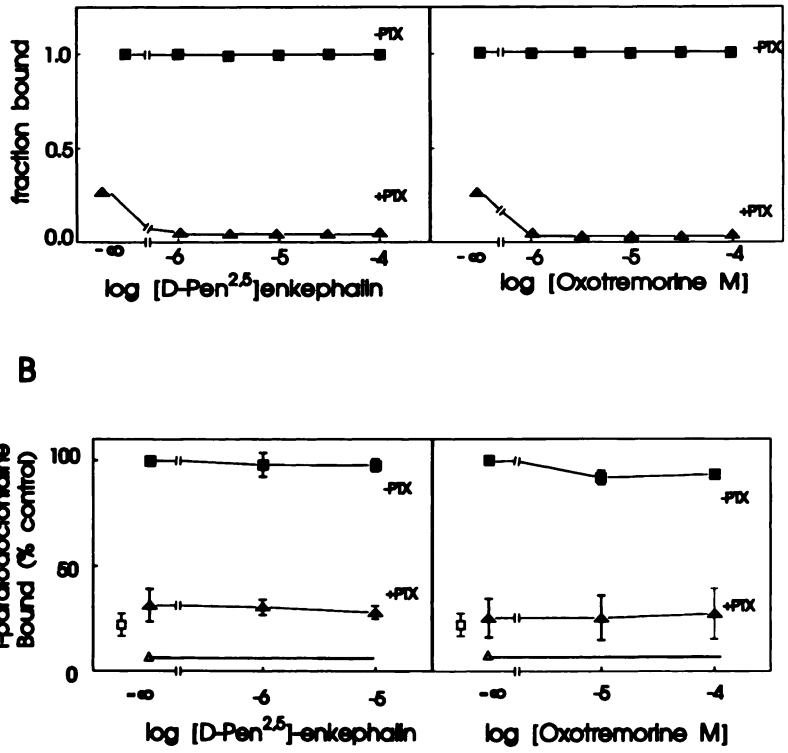

Fig. 5. Effect of muscarinic or opiate agonists on [^{125}I]PIC binding to the α_2-adrenergic receptor. Theoretical (A) and observed (B) effects of muscarinic and opiate agonists on ^{125}I-PIC binding are shown. A, Theoretical simulations of PIC binding to the α_2-adrenergic receptor in NG108–15 membrane vesicles were calculated as described in Experimental Procedures. The effect of pertussis toxin was simulated by decreasing the concentration of G protein until ligand binding was reduced to 25% of control. G protein concentration was 50-fold greater than α_2-adrenergic receptor concentration (see Table 1) for control calculations and was reduced to one-fourth that of α_2-adrenergic receptor to simulate pertussis toxin treatment. All other constants were the same as for previous simulations. B, Radioligand binding assays were performed as described in Experimental Procedures. Before membrane preparation, cells were treated with either vehicle (*−PTX*) (■) or 10 ng/ml pertussis toxin (*+PTX*) (▲) for sufficient time to reduce agonist binding to approximately 25% of control, as described in Experimental Procedures. Membranes were incubated with 1 nM [^{125}I]PIC for 45 min in 200-μl total volume. Specific [^{125}I]PIC binding in the presence of 50 μM Gpp(NH)p is also indicated (□, control; △, plus pertussis toxin). Nonspecific binding was determined in the presence of 10 μM yohimbine and was 10–15% of control. Data are the average of three experiments, plotted as mean ± standard error.

of [D-Pen^{2,5}]enkephalin. However, this effect is not reversed by the opiate antagonist naltrexone and is, therefore, not dependent on agonist binding to the opiate receptor (i.e., it appears to be a nonspecific effect). Thus, there is no evidence for binding cross-talk among α_2-adrenergic, muscarinic, and opiate receptors in NG108–15 cell membranes.

Discussion

We (14, 28) and others (13, 29, 30) have pointed out that the simple TCM cannot account for the complex behavior of agonist binding when there is an excess of G protein. Receptor heterogeneity or compartmentation was proposed to account for the biphasic agonist binding (14, 28). In this paper, we have expanded the simple TCM to include multiple receptors and G proteins. Even with this more complex model, it does not appear that the TCM adequately predicts the high and low affinity agonist binding observed. In addition, the lack of binding cross-talk among multiple receptors known to interact with the same G protein suggests that compartmentation rather than receptor heterogeneity is the likely mechanism to account for biphasic agonist binding.

In this paper we outline the theory of agonist binding in the 2R1G and 1R2G models. As expected, two receptors coupling to the same G protein should exhibit heterologous binding interactions but only if the G protein is limiting. It is surprising

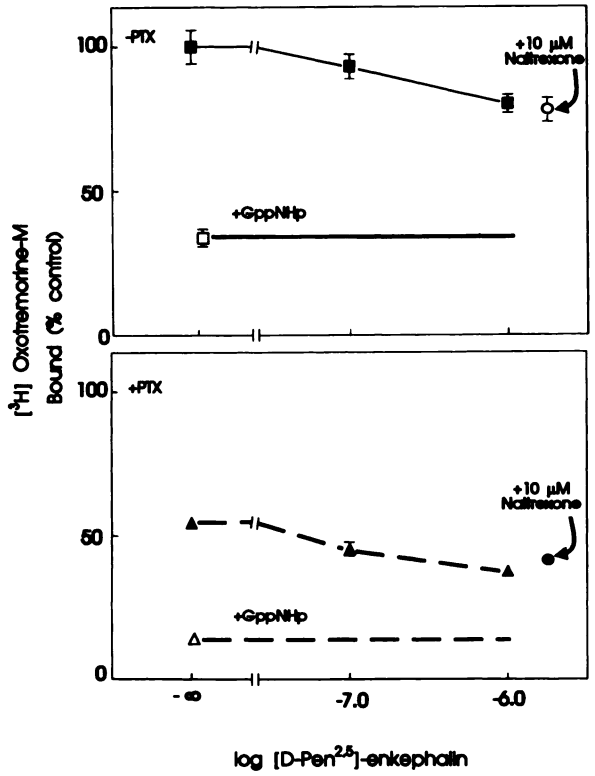

Fig. 6. Effect of opiate agonist on [^{3}H]oxo M binding to the muscarinic receptor. The effect of [D-Pen^{2,5}]enkephalin (δ-opiate agonist) on [^{3}H]oxo M binding is shown. Radioligand binding assays were performed as described in Experimental Procedures. Before membrane preparation, cells were treated with either vehicle (*–PTX*) (■) or 10 ng/ml pertussis toxin (*+PTX*) (▲) for sufficient time to reduce agonist binding to approximately 25% of control, as described in Experimental Procedures. Membranes were incubated with 1 nM [^{3}H]oxo M for 90 min in 500-μl total volume. The effect of 10 μM naltrexone plus 10 μM [D-Pen^{2,5}]enkephalin is also indicated (○, without pertussis toxin; ●, plus pertussis toxin). Note that, although [D-Pen^{2,5}]enkephalin does reduce [^{3}H]oxo M binding, the effect is not reversed by 10 μM naltrexone (opiate antagonist) and is, therefore, presumably nonspecific. [^{3}H]Oxo M binding in the presence of 50 μM Gpp(NH)p is indicated (□, without pertussis toxin; △, plus pertussis toxin). Nonspecific binding was determined in the presence of 10 μM atropine and was 40–50% of control. Data are from three experiments, plotted as mean ± standard error. Theoretical simulations showed results similar to those in Fig. 5 (data not shown).

how stringent the conditions are for biphasic agonist binding to be produced in the 2R1G model. For a second receptor to deplete the correct amount of G protein to induce two affinities for the other receptor there must be a very high affinity of the second receptor for G protein (as when an agonist is present). Also, the amount of the second receptor must be very close to the amount of the G protein (i.e., within <5% in our results). The routine observation of high and low affinity agonist binding with almost all G protein-coupled receptors mitigates against such a precise stoichiometry in the mechanism.

In the NG108–15 cells, the lack of cross-talk between opiate and α_2 receptors was particularly surprising because 1) both opiate and α_2 receptors couple efficiently to the G_{i2} subtype of G_i (19–21) and 2) G_i levels were reduced to a limiting level by pertussis toxin pretreatment, which should have favored cross-talk. Muscarinic and opiate receptors both outnumber the α_2-adrenergic receptor and the opiate receptors outnumber the muscarinic, so it should have been possible to deprive either the α_2-adrenergic or the muscarinic receptor of G proteins. Our data show no evidence of any receptor-G protein cross-talk in NG108–15 membranes during equilibrium agonist binding assays, in spite of the prediction of the 2R1G model that cross-talk should occur after pertussis toxin pretreatment. A single instance of binding cross-talk between G_i-coupled receptors has been reported in the literature. Härfstrand *et al* (31) found that α_2-adrenergic agonists reduced the binding of neuropeptide Y in brain membranes. The effect was quite small (~10%) and reversibility by antagonists was not reported, so nonspecific effects (as seen in Fig. 6) must be considered.

There are three classes of mechanisms that could account for the lack of binding cross-talk among the α_2-adrenergic, muscarinic, and opiate receptors. First, the modeling may not use appropriate parameters. Second, subtype differences among the G proteins may result in distinct pools that interact selectively with the different receptors. Third, compartmentation may prevent free movement of the receptors and G proteins in the membranes and/or cells. As we consider these models, we should also look at other data that must be compatible with our model.

One reason for the divergence of theory and experiment may be that incorrect affinity constants and concentrations of the various species were used. This is unlikely for several reasons. First, G protein is present in excess over receptor in several cell types, including S49 lymphoma, neutrophils, and platelets (14–16). The G_{i2} subtype has also been shown to be present in picomole per milligram quantities in NG108–15 membranes, outnumbering all three receptor types (32). The G protein subtype would only have to be present in a slightly higher concentration (2–3-fold higher) for the TCM to predict all high affinity agonist binding (14). We assumed that the concentration of G protein was reduced by about 100-fold after pertussis toxin treatment, so the functional effect of the ribosylation [i.e., the reduction of Gpp(NH)p-sensitive binding] is the same for both the experimental data and the theoretical simulations. This indicates that we have made the G protein functionally limiting with respect to agonist binding and thus should have produced cross-talk among the receptors. Finally, the qualitative behavior of ligand binding is relatively constant over a wide range of affinity rate constants, so that even relatively large errors in estimating the constants (100-fold) would not affect the qualitative results.

A previous theoretical model, described by Kenakin and Morgan (33), showed that cross-talk was possible among two receptor types and two G proteins. That model examined effector production instead of agonist binding and the authors used a single receptor that behaved like two different receptor types. In the model of Kenakin and Morgan, if G protein was limiting, then the receptor type with the greater affinity for G protein could steal G protein from the receptor type with lower affinity. The presence of the second receptor type alone was not enough to alter effector production. This shows that our theoretical results are not peculiar to agonist binding but can be extended to effector production.

G protein subtypes could represent the distinct pools of G protein accessed by α_2-adrenergic and opiate receptors. A recent study has shown that δ-opiate receptors in NG108–15 cells are coupled to three different pertussis toxin-sensitive substrates (34). Having three types of G proteins coupling to the δ receptor might potentially mask cross-talk between the δ-opiate receptor and the muscarinic and α_2-adrenergic receptors, because the

opiate receptor would have another source of G proteins that might be unavailable to the other two receptors. Because agonist binding to the α_2 receptor is largely dependent on the G_{i2} subtype (20) and the opiate receptor is clearly capable of binding to G_{i2} (21, 34), we would still expect opiate agonist-mediated inhibition of α_2 agonist binding. Furthermore, cross-talk is clearly possible on a physiological level among all three receptors in NG108–15 cells (24), as well as in other cell types (35, 36). In addition to α subunit heterogeneity, there are also β and γ subunit subtypes (37, 38). The β_{35} and β_{36} subtypes are very widely distributed and there is no known specificity to their interactions with receptors (37).[4] As more γ subunits are found (38–41) it is intriguing that they may provide more specificity to G protein-receptor interactions, but we do not have any data to directly support or refute this hypothesis.

The final class of mechanisms for lack of cross-talk among these receptors would be limitations of access of the receptors to G proteins and vice versa. We can see this by examining the assumptions of the 2R1G model. The first assumption is that receptors and G proteins can move freely through a simple three-dimensional solution, instead of a complex two-dimensional lipid bilayer with multiple protein components. Limitations of access could be produced by the following factors: 1) the plasma membrane could be broken into fragments that are so small that there is only one type of receptor in each membrane vesicle, 2) the receptors could reside in different subcellular fractions, or 3) receptors and G proteins may be in the same bilayer but not move freely in it.

Estimates of the size of vesicles produced from NG108–15 cells, together with the receptor density and cell size, suggest that there would be at least 3 opiate receptors, 1.5 muscarinic receptors, and 1 α_2 receptor per vesicle. Thus, there would be very few vesicles (~1%)[5] that would by random chance contain α_2-adrenergic receptors but completely lack opiate and muscarinic receptors. Distinct subcellular fractions are possible and have been suggested for neuroblastoma cells (42). This would be unlikely to correspond to extracellular and intracellular membranes, because most receptors are on the cell surface. Organization of the plasma membranes into topographic regions that are selectively enriched in certain receptors is the rule in neuronal cells, with their highly differentiated morphology. Although it may not be so obvious, the undifferentiated NG108–15 cells could also have segregation of the different receptors into macroscopic regions. This, of course, would require either limitations to diffusion of the receptors and G proteins (see below) or strong gradients of insertion or removal of receptors on the surface. The latter is supported by evidence that shows that proteins are sent specifically to either apical or basolateral membrane surfaces in polarized epithelial cells (43).

Another assumption of the TCM is that agonist binding using purified plasma membranes represents a true equilibrium. Exchange of receptors and G proteins may be kinetically limited when no GTP is present, as in radioligand binding assays. If receptors and G proteins are normally tightly coupled and dissociate only in the presence of GTP, then in the absence of GTP the system may not be at a true equilibrium. Furthermore, no cross-talk would be observed with tightly coupled receptors and G proteins, because the G proteins would not be able to exchange from one receptor to another. There is evidence that receptors and G proteins are precoupled before agonist binding (13, 28, 44), which would support this hypothesis. Our data showing lack of cross-talk during equilibrium agonist binding is also consistent with tight coupling; however, it cannot explain why agonists bind receptors with two apparent affinities in the presence of excess G protein.

Finally, compartmentation may prevent free movement of the receptors and G proteins in the membranes and/or cells. If some receptors were in compartments that lack G protein and others were in contact with a G protein population inaccessible to other receptor types, it could explain both high and low affinity agonist binding and the lack of cross-talk in equilibrium binding assays. Experimental evidence has been found for the sequestration of membrane proteins in compartments (45). Furthermore, patch-clamp experiments on bullfrog sympathetic neurons show that, although the muscarinic, substance P, and luteinizing hormone receptors are all coupled through a pertussis-sensitive G protein, each binds to a separate pool of G proteins (46). This was shown by agonist-enhanced guanosine-5′-*O*-(β-thio)diphosphate binding, in which each agonist blocked only its own effect, whereas effects through the other two receptors were not blocked.

The physical basis of such compartmentation could be 1) fluid and nonfluid regions of lipid, which only slowly interchange, or 2) tight binding of G protein subunits to protein components such as the cytoskeleton. The β-subunit of the G protein has been shown to be sequestered in the Triton X-100-insoluble fraction of S49 lymphoma cells, which is thought to be composed primarily of cytoskeleton (47). Nakamura and Rodbell (48, 49) have identified large molecular weight complexes containing G protein subunits. Also, we have recently shown in fluorescence photobleaching recovery experiments that the mobility of G protein $\beta\gamma$ subunits is markedly limited in NG108–15 cells (50, 51). Approximately 84% of the $\beta\gamma$ subunit is immobile and 66% of the α subunit is immobile. The greater mobility of the α subunit may help explain why cross-talk in activation of effectors is common, whereas we see no cross-talk in binding. In fact, Peters (52) proposed limited mobility of G proteins in membranes (e.g., $\beta\gamma$ subunits), with higher mobility in cytosolic fractions (e.g., α subunits). Our photobleaching data provide a plausible mechanism for this idea.

Thus, we have shown that the TCM cannot explain the binding behavior of multiple receptor-G protein systems. Although originally proposed to describe equilibrium agonist binding to a single receptor in a GTP-free system, the model cannot even accurately describe such a simple system. Furthermore, the expanded model does not correctly predict the lack of cross-talk between multiple ligands in agonist binding to purified plasma membranes. The problem appears to lie in the assumption made by the model of the free diffusability of receptors and G proteins and the existence of a true equilibrium between different receptor states in a GTP-free system. Substantial biochemical and biophysical data from our laboratory and others suggest that the simple concept of receptors and G proteins floating freely in a Singer-Nicholson sea of lipid is not adequate.

[4] A recent paper raises the intriguing possibility that β subunits do convey specificity to receptor-G protein interactions (53).

[5] For random discrete events, the probability that there are no occurances is governed by a Poisson distribution. If there are, on average, m events then the chance of no events occurring is $P_0 = e^{-m}$. For an average of 3 receptors/vesicle (opiate) P_0 is 0.0498, and for 4.5 receptors/vesicle (opiate plus muscarinic) P_0 is 0.011.

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Appendix A

In the 2R1G model, the G protein binds to receptor R_1 or R_2 with a given affinity (K_4 and K_{4b}) to produce an RG complex. Agonist can bind to the RG complex with high affinity (K_3 and K_{3b}). Because D_1 is defined to be the radioligand, only binding of D_1 to R_1 is measured. Agonist binds with low affinity (K_1 and K_{1b}) when receptor is alone. Furthermore, for some calculations constants were different for R_1 and R_2. The two receptors compete for a single pool of G protein. When enough receptor is present, the less numerous receptor becomes deprived of G protein and cross-talk is observed as a reduction in agonist binding. The theoretical calculations described in this paper were performed using the following equations, which describe the interactions among agonist(s), receptor(s), and G protein(s) at equilibrium. As noted above, receptors and G proteins are assumed to diffuse freely through the membrane and to interact without hindrance. R_{1T} is the total concentration of the first receptor and R_1 is the free concentration of that receptor; D_1 is the free concentration of drug binding to the first receptor. R_{2T} is the total concentration of the second receptor and R_2 is the free concentration of that receptor; D_2 is the free concentration of the drug binding to the second receptor. G_T is the total concentration of G protein and G is the uncomplexed concentration. Concentrations of the various molecular complexes formed are as noted.

We can write the following conservation equations:

$$R_{1T} = R_1 + D_1R_1 + D_1R_1G + R_1G$$

$$R_{2T} = R_2 + D_2R_2 + D_2R_2G + R_2G$$

$$G_T = G + R_1G + R_2G + D_1R_1G + D_2R_2G$$

The ligand, receptor and G protein interactions are governed by these equilibrium equations.

$$K_1 = \frac{D_1 \cdot R_1}{D_1R_1}$$

$$K_2 = \frac{D_1R_1 \cdot G}{D_1R_1G}$$

$$K_3 = \frac{D_1 \cdot R_1G}{D_1R_1G}$$

$$K_4 = \frac{R_1 \cdot G}{R_1G}$$

$$K_{1b} = \frac{D_2 \cdot R_2}{D_2R_2}$$

$$K_{2b} = \frac{D_2R_2 \cdot G}{D_2R_2G}$$

$$K_{3b} = \frac{D_2 \cdot R_2G}{D_2R_2G}$$

$$K_{4b} = \frac{R_2 \cdot G}{R_2G}$$

These equations were solved by combining them and calculating the free concentration of G protein using eq. 1 below. Equation 1 was solved using Newton's method of estimating polynomial roots. Once the free concentration of G protein (x in eq. 1) was calculated, the concentrations of the various receptor species were also calculated from the equilibrium and conservation equations. From this, the amount of agonist bound to receptor was determined (see below).

$$ax^3 + bx^2 + cx + d = 0 \quad (1)$$

$$a = \delta_1 \cdot \delta_2$$

$$b = (\delta_1 \cdot \delta_2)(R_{1T} + R_{2T} + -G_T) + (\kappa_1 \cdot \delta_2) + (\kappa_2 \cdot \delta_1)$$

$$c = (\delta_1 \cdot \kappa_2 \cdot R_{1T}) + (\delta_2 \cdot \kappa_1 \cdot R_{2T}) + (\kappa_1 \cdot \kappa_2) - [(\delta_1 \cdot \kappa_2) + (\delta_2 \cdot \kappa_1)G_T]$$

$$d = -G_T \cdot \kappa_1 \cdot \kappa_2$$

$$x = \textit{free concentration of G protein}$$

where

$$\delta_1 = 1/K_4 + (D_1/K_4 \cdot K_3)$$

$$\delta_2 = 1/K_{4b} + (D_2/K_{4b} \cdot K_{3b})$$

$$\kappa_1 = 1 + (D_1/K_1)$$

$$\kappa_2 = 1 + (D_2/K_{1b})$$

A range of affinity values were used for calculations; K_1 and K_{1b} varied between 10^{-5} and 10^{-11} M, whereas all other constants varied between 10^{-7} and 10^{-12} M.

Appendix B

Similar models and theoretical predictions can made for the interaction of one receptor and multiple G proteins as for the interaction of two receptors with one pool of G protein. The 1R2G model (Fig. 7) describes the interaction of a single receptor population with two G protein populations. As in the TCM and 2R1G model, agonist binds to receptor alone with low affinity and to receptor coupled to either G protein with

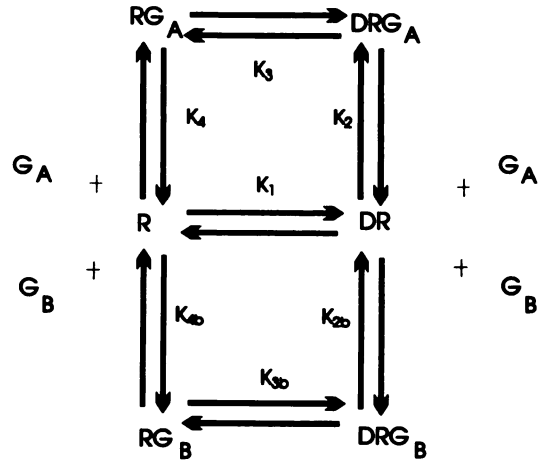

Fig. 7. 1R2G model with two G proteins coupling to one receptor. The 1R2G model is a modification of the TCM that describes the interactions between two G protein types (G_a and G_b) and one receptor (R) and the effects of this interaction on the binding of agonist (D) to R. As in the 2R1G model (see Fig. 1), the system is at equilibrium and contains no guanine nucleotides. See Appendix B for details of the model and simulations.

high affinity. G_{aT} is the total concentration of the G protein G_a, and G_a is the free concentration of that G protein. G_{bT} is the total concentration of the G protein G_b, and G_b is the free concentration. R_T is the total concentration of receptor, and R is the free concentration of receptor. D is the free concentration of drug.

$$K_1 = \frac{D \cdot R}{DR}$$

$$K_{2a} = \frac{DR \cdot G_a}{DRG_a}$$

$$K_{3a} = \frac{D \cdot RG_a}{DRG_a}$$

$$K_{4a} = \frac{R \cdot G_a}{RG_a}$$

$$K_{2b} = \frac{DR \cdot G_b}{DRG_b}$$

$$K_{3b} = \frac{D \cdot RG_b}{DRG_b}$$

$$K_{4b} = \frac{R \cdot G_b}{RG_b}$$

Conservation and equilibrium equations were derived for the two-G protein model and were used to solve for the free concentration of receptor. Once the free concentration of receptor is known, it can be used to calculate the amount of drug bound to the various receptor species. The free concentration of receptor in this model is also represented by a cubic equation of the same form as eq. (1) where

$$a = \beta_1 \cdot \beta_2 \cdot \gamma$$

$$b = [(G_{bT} + G_{aT} - R_T)(\beta_1 \cdot \beta_2)] + [\gamma \cdot (\beta_1 + \beta_2)]$$

$$c = \gamma - [R_T \cdot (\beta_1 + \beta_2)] + (G_{aT} \cdot \beta_1) + (G_{bT} \cdot \beta_2)$$

and

$$\beta_1 = 1/K_{4a} + D/(K_{3a} \cdot K_{4a})$$

$$\beta_2 = 1/K_{4b} + D/(K_{3b} \cdot K_{4b})$$

$$\gamma = 1 + D/K_1$$

Note that there are only seven equilibrium constants in the two-G protein model because the affinity of drug for receptor alone is not affected by the G protein present.

For these simulations, both G proteins could couple to receptor and cause receptor to bind agonist with the same high affinity, regardless of which G protein was bound. The concentration of the first G protein, G_a, was assumed to be equal to that of receptor (R), whereas the concentration of the second G protein, G_b, was 100-fold higher than that of receptor. Increasing K_{4b} (dissociation constant for G_b binding to receptor) decreased the affinity of agonist binding to receptor, as shown by the rightward shifts in agonist binding (Fig. 8A). Fig. 8B shows the decrease in affinity of agonist binding as measured by the K_d. Simply having a large population of different G proteins is clearly not sufficient to alter agonist binding to receptor, because G protein affinity is also important.

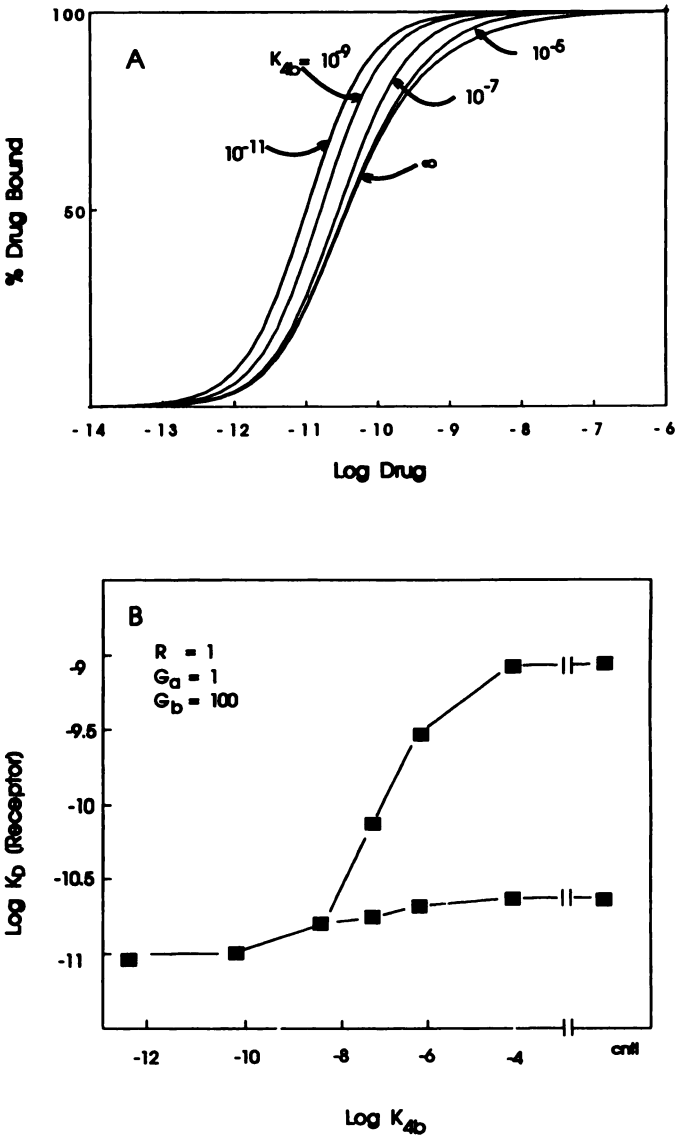

Fig. 8. Effect of two receptor types coupling to the same G protein. Simulations of the 1R2G model were done as described in Experimental Procedures and Appendix B (see Fig. 7). Dissociation constants for ligand binding to receptor were calculated from the theoretical data as described in Experimental Procedures. The concentration of R was 10^{-11} M. G_a concentration was 10^{-11} M; G_b was 10^{-9} M. K_d for G_a and receptor was 10^{-11} M. A, Each *curve* represents binding of ligand to receptor with varying K_d for receptor-G_b interactions. Note that increasing the dissociation constant (K_d) for G_b and receptor decreased the overall affinity of agonist binding. B, Each *data point* represents the dissociation constant for ligand and receptor at a given K_d for receptor-G_b interactions. *Double points* mean that two affinities and nonhyperbolic ligand binding were seen.

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