

Activation of Guanosine 5'-[γ - 35 S]thio-triphosphate Binding through M₁ Muscarinic Receptors in Transfected Chinese Hamster Ovary Cell Membranes: 2. Testing the "Two-States" Model of Receptor Activation

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ABSTRACT

I suggested in the accompanying article [*Mol Pharmacol* 2001; 59:875-885] that muscarinic receptors catalyzed G protein activation. Acetylcholine or carbamylcholine recognition facilitated not only the GDP release from receptor-coupled inactive G proteins but also the release of G_{GTP γ S} from the (unstable) HRG_{GTP γ S} complex. The two effects facilitated [35 S]GTP γ S binding in the presence of GDP, but could be studied separately by comparing [35 S]GTP γ S binding in the absence and presence of GTP. Guanyl nucleotides affected the efficiency of receptor-G protein coupling. The relative efficacies of partial agonists in the absence and presence of GTP should remain nonlinearly correlated if all agonists stabilize (to different ex-

tents) the same active receptor conformation. The correlation between M₁ muscarinic agonists' efficacy in accelerating [35 S]GTP γ S binding in the absence of other nucleotides and their in vivo efficacy (inositol phosphate accumulation) was in fact very poor. This probably reflected the presence of GTP in intact cells: pertussis toxin pretreatment (which inactivates the G_{i/o} proteins) did not affect the agonists' efficacy profile (evaluated in the absence of spare receptors), but the addition of GTP to the [35 S]GTP γ S binding medium did. These results did not support the allosteric "two states" model of receptor activation, but suggested that different agonists induced different receptor conformations ("induced fit").

Small ligands are able to induce significant conformational changes in their binding proteins. Two extreme descriptions of this phenomenon can be seen by analogy with the hemoglobin and steroid receptors ligand-induced conformational changes, respectively. Hemoglobin exists in two dominant conformations: an empty conformation (T) stabilized by a network of ionic and hydrogen bonds, and another, oxygen-bound conformation (R). Allosteric modulators, which recognize a single binding site between the four subunits, do not affect the "R" and "T" hemoglobin conformations but stabilize one of the two states. They thereby inhibit or facilitate oxygen binding. The estrogen receptor conformation depends on the nature of the bound ligand: different compounds induce different receptor conformations and therefore produce variable biological effect patterns (Smith and O'Malley, 1999).

G-protein coupled receptors (GPCRs) possess seven trans-

membrane α -helices. Such α -helices are typically stable and rigid: they are stabilized by an extended of hydrogen bonds network. GPCR activation is thought to involve the disruption of an intramolecular ionic bridge and coordinated movement of two or more transmembrane helices (Sealfon et al., 1995; Porter et al., 1996; Gether et al., 1997; Scheer et al., 1997; Kobilka et al., 1999; Sheikh et al., 1999; Ghanouni et al., 2000; Porter and Perez 2000; Schulz et al., 2000). In addition, some GPCRs dimerize, and this affects their ability to activate G proteins (Hebert et al., 1998; Hebert and Bouvier 1998). It therefore seemed plausible that—like hemoglobin—GPCRs possess two predominant conformations. Agonists can be defined as ligands that stabilize an active receptor conformation and thereby increase G protein activity, whereas inverse agonists, which recognize preferentially a "resting" receptor conformation, suppress the basal G protein activity (Samama et al., 1993, 1994; Chidiac et al., 1994).

In response to agonist binding, GPCRs usually activate one or several trimeric G protein(s), receptor kinases known as GPCR kinases (Pitcher et al., 1998), and "Velcro" proteins, such as arrestins (Krupnick and Benovic, 1998) that are

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ABBREVIATIONS: GPCR, G protein-coupled receptors; GTP γ S, guanosine 5'-thio-triphosphate; 4-DAMP mustard, [4-diphenylacetoxy-1-(2-chloroethyl) piperidine]; [3 H]NMS, L-[N-methyl- 3 H]scopolamine methyl chloride; CHO, Chinese hamster ovary cells; IP, inositol phosphate; DMEM, Dulbecco's minimum essential medium; G_{GTP}, GTP-bound G proteins; G_{GTP γ S}, GTP γ S-bound G proteins.

involved in the desensitization of the functional response and receptor internalization, respectively. The mode of receptor activation (induced fit or stabilization of the one-and-only active conformation?) might have important repercussions on the receptor-intracellular protein interaction: if only two receptor conformations exist, agonists and inverse agonists should activate or inhibit, respectively, the same set of intracellular proteins with the same relative efficacy; if each ligand induces a distinct receptor conformation, one might expect to find drugs with different G protein, GPCR kinase, and arrestin activation profiles.

Several results, in fact, suggest that the conformation of some GPCRs is ligand-dependent. First, different ligands may induce different G protein activation profiles (Meller et al., 1992; Spengler et al., 1993; Robb et al., 1994; Perez et al., 1996; Berg et al., 1998; Bonhaus et al., 1998; Zuscik et al., 1998). Second, the ligands' abilities to induce receptor phosphorylation, desensitization, and internalization are not always correlated with their intrinsic activities for G protein activation (Keith et al., 1996; Blake et al., 1997; Roettger et al., 1997; Yu et al., 1997; Mhaouty-Kodja et al., 1999). Third, the rate of activated G protein release by β_2 -adrenergic receptors depends on the bound agonist (Krumins and Barber 1997). Fourth, the efficacy of ligands stimulating adenylate cyclase through β_2 -receptor-G_s fusion proteins in the presence of GTP differed considerably from their efficacies in the presence of XTP or ITP (Seifert et al., 1999).

My goal was to test whether different muscarinic agonists stabilize (to different extents) the same unique "active" M₁ receptor conformation or induce different conformational changes. I designed two experimental approaches: 1) G protein activation is a cyclic reaction (Cassel and Selinger 1978; Hamm 1998). Reaction cycles cannot occur faster than the slowest reaction in the cycle, known as the "rate-limiting step". It is possible to switch the rate-limiting step for G protein activation by changing the guanyl nucleotide composition of the incubation medium. I verified that on theoretical grounds, if all muscarinic agonists stabilize the same receptor conformation, the rank order of agonist efficacy should be independent of the incubation conditions (Appendix¹); then I investigated the effect of guanyl nucleotides on the rank order of efficacy of a panel of muscarinic agonists. 2) M₁ muscarinic receptors facilitate [³⁵S]GTP γ S binding to G_{q/11} and G_{i/o} proteins (Lazareno and Birdsall 1993; DeLapp et al., 1999). I tested the effect of pertussis toxin treatment (which inactivates G_{i/o} proteins) on the agonists' efficacy profile in [³⁵S]GTP γ S binding assays. My results did not support the two-states model of G protein activation.

Experimental Procedures

Materials. 4-Diphenylacetoxyl-1-(2-chloroethyl)piperidine (4-DAMP) mustard was a generous gift from Dr. R. Barlow (Kirkby Stephen, Cumbria, UK). *l*-[N-methyl-³H] scopolamine methyl chloride ([³H]NMS; 80 Ci/mmol) and guanosine 5'-[γ -³⁵S]thio-triphosphate, triethylamine salt ([³⁵S]GTP γ S; >1000 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Bucks, UK). Unlabeled guanyl nucleotides (as Li⁺ salts) were obtained from Roche Molecular Biochemicals (Mannheim, Germany). The agonists were obtained from the following sources: acetylcholine chloride, (rac)-acetyl-(β -

methyl)choline bromide, acetylthiocholine chloride, arecoline hydrobromide, and (rac)-carbamyl-(β -methyl)choline chloride were from Sigma Chemical Co. (St. Louis, MO), carbamylcholine hydrochloride was obtained from Merck (Darmstadt, Germany), oxotremorine sesquifumarate was from Aldrich (Milwaukee, WI), oxotremorine methiodide was from ICN Biomedicals Inc. (Aurora, OH) and pilocarpine hydrochloride was from Janssen Chimica (Beerse, Belgium). Pertussis toxin was obtained from Sigma. All other chemicals were of the highest grade available. Stably transfected CHO cells expressing the human M₁ muscarinic receptor subtype (Hm1 CHO cells) were a generous gift from Dr. N. Buckley (London, England).

Methods. The Hm1 CHO cell culture conditions, 4-DAMP mustard and pertussis toxin treatments, [³⁵S]GTP γ S and [³H]NMS binding experiments were performed as detailed in the accompanying article (Waelbroeck, 2001).

Inositol Phosphates Synthesis and [³H]NMS Binding to Intact Cells. Hm1 CHO cells were subcultured for 48 to 72 h in 24-well plates, in the presence (for IP turnover studies) or absence (for binding studies) of [³H]inositol (1 μ Ci/ml). Unless otherwise indicated, confluent cells were treated 1 h with 30 nM 4-DAMP mustard before functional studies.

The inositol phosphates accumulation was measured as follows. Each well was incubated 30 min at 37°C with 250 μ l of DMEM enriched with myo-[³H]inositol (1 μ Ci/ml), 20 μ l of 150 mM LiCl (final concentration, 10 mM), and 30 μ l of agonist solution in the HEPES/NaCl-MgCl₂ binding buffer. The medium was then aspirated, the incubation stopped by 0.5 ml of ice-cold methanol, and the cells scraped. Each well was rinsed by a second 0.5-ml methanol addition. The pooled fractions were extracted by 2 ml of chloroform in the presence of 1 ml of water. A 1.5-ml fraction of the aqueous phase was deposited on ion exchange column to separate [³H]inositol from the [³H]inositol phosphates (IP, IP₂, IP₃, and IP₄) as described previously (Van-Rampelbergh et al., 1997). The total radioactivity was measured by liquid scintillation counting.

[³H]NMS binding was measured in a larger incubation volume to minimize tracer depletion by muscarinic receptors. Each well was incubated for 30 min at 37°C with 1.25 ml of DMEM enriched with [³H]NMS (0.8 nM), 100 μ l of 150 mM LiCl (final concentration, 10 mM) and 150 μ l of agonist solution in the HEPES/NaCl-MgCl₂ binding buffer. The medium was then aspirated, the wells rinsed with ice-cold DMEM, and the cells harvested in 1 ml of ice-cold PBS (phosphate-buffered saline) enriched with 1 mM EDTA. Each well was rinsed with 1 ml PBS/EDTA, the two fractions were pooled, and the bound radioactivity measured by liquid scintillation counting. The tracer's affinity (K_D , 0.30 ± 0.06 nM) was determined in the same incubation conditions by saturation curves (0.03 to 2.0 nM [³H]NMS). I verified the validity of the Cheng and Prusoff (1973) equation by analysis of [³H]NMS/agonist competition curves at different tracer concentrations.

Data Analysis. Non linear curve fitting was performed with a computer assisted curve fitting program (Prism; GraphPAD Software, San Diego, CA).

Results

Agonist Binding and Functional Properties in CHO Cell Membranes. I verified that agonists achieved equilibrium binding to CHO cell membranes within 10 min [accompanying article (Waelbroeck, 2001)]. I then compared the agonist binding properties in the absence and presence of guanyl nucleotides with their functional properties in the same membranes (see Tables 1 and 2). In the absence of guanyl nucleotides, the full and "almost full" agonists (efficacy > 80% of acetylcholine, see Table 1) recognized 25 to 30% of the binding sites with a high affinity and had a significantly lower affinity for the remaining [³H]NMS binding sites (Table 2). The partial agonist competition curve

¹ Because of the length of the Appendix, it is not printed herein. It can be found in its entirety in the online version of this article.

fitting was not significantly improved by using a two-sites model: I report a single "corrected IC_{50} " value (using a log scale) in Table 1. These values are identical to the agonists' K_D values only if they indeed recognize a single site. I observed, however, that all agonists had a lower affinity in the presence of GTP γ S ($\geq 1 \mu M$), GTP ($\geq 1 \mu M$) or GDP ($\geq 3 \mu M$) than in their absence (Table 1): it is likely that, like full agonists, partial agonists discriminated two receptor states (HR and HRG) in the absence of guanyl nucleotides. It is indeed known that the existence of two receptor states is very difficult to demonstrate if the proportion of high- or low-affinity sites or their affinity ratio is too low (De Lean et al., 1982).

All the compounds studied stimulated [35 S]GTP γ S binding in the absence and in the presence of GDP. Their potencies (pEC_{50} values) and efficacies (E_{max} , expressed in percentage of the maximal response to acetylcholine) are summarized in Table 1. In the absence of unlabeled guanyl nucleotides, the agonist concentrations necessary for half-maximal [35 S]GTP γ S binding stimulation (EC_{50} values) were lower than the concentrations necessary for occupancy of half of the receptors (corrected IC_{50} values). As shown in the accompanying article (Waelbroeck, 2001), this was not because of spare receptors: the high-affinity agonist-receptor complexes were more efficient than the low-affinity complexes for [35 S]GTP γ S binding stimulation in the absence of competing nucleotides. The agonists' EC_{50} and binding K_D values were well correlated in the presence of $3 \mu M$ GDP (Table 1).

Agonists Binding and Functional Properties in Intact Cells. [3H]NMS recognized a single binding site population with a $K_D = 300$ pM at $37^\circ C$, in the Dulbecco's minimal essential medium. I did not observe biphasic agonist competition curves in intact cells: all the agonists studied recognized a single receptor state (not shown). The agonist K_D values in intact cells at $37^\circ C$ (summarized in Table 3) were, as a rule, similar to the "low-affinity" K_D values observed in membranes at $30^\circ C$.

Carbamylcholine and pilocarpine stimulated 10 to 20 fold the inositol phosphates synthesis in intact cells (Fig. 1). Pretreatment with 30 nM 4-DAMP mustard did not affect the [3H]NMS affinity, suggesting that there was no significant competitive inhibition by residual antagonists after this procedure (not shown). I observed a marked (85–90%) decrease of the total [3H]NMS receptor concentration (not shown). The agonist dose effect curves were shifted to significantly higher

concentrations (Fig. 1). In addition, the maximal effect of the partial agonist, pilocarpine, (but not of the full agonist, carbamylcholine), decreased (Fig. 1). Pretreatment with higher 4-DAMP mustard concentrations or longer pretreatments did not result in a significantly greater decrease of the residual muscarinic receptor concentration (not shown): the residual muscarinic receptor concentration probably reflected the new equilibrium between receptor inactivation and resynthesis.

The agonists K_D values, and their EC_{50} and efficacies relative to acetylcholine (in 4-DAMP mustard-treated cells) are summarized in Table 3. The partial agonists of EC_{50} and K_D values were very similar (Table 3) and the maximal effect induced by these agonists decreased with decreasing receptor concentrations (Fig. 1 and results not shown). I therefore feel confident that these agonists had no spare receptors, and that their EC_{50} values were near the concentrations necessary for half-maximal receptor activation, K_{act} . In contrast, decreasing the receptor density did not affect the maximal response to full agonists. The small difference between the full agonists' EC_{50} and K_D values in intact cells probably reflected the presence of some residual spare receptors for these compounds.

Effect of the Incubation Conditions on the Agonists' Efficacies. The agonists' efficacies in intact cells were poorly correlated with their efficacies on membranes in the absence of unlabeled nucleotides (Fig. 2A). I can provide two explanations for this discrepancy: 1) GTP is present in very large concentrations in intact cells, but absent from the [35 S]GTP γ S binding buffer: this affects the rate-limiting step of the G protein activation cycle [accompanying article (Waelbroeck, 2001)]. Partial agonists might differ in their relative abilities to stimulate the ternary complex formation as opposed to activated G protein release. 2) It has been demonstrated that muscarinic agonists stimulate [35 S]GTP γ S binding to $G_{q/11}$ as well as to $G_{i/o}$ proteins (Lazareno and Birdsall 1993; DeLapp et al., 1999). $G_{i/o}$ proteins poorly stimulate the phospholipase C: should agonists differ in their abilities to stimulate the different G protein subtypes, those that activate preferentially $G_{q/11}$ proteins must be relatively more efficient in the inositol phosphate turnover assay. To test these two hypotheses, I 1) compared [35 S]GTP γ S binding in the absence and presence of unlabeled nucleotides (Fig. 2B), and 2) studied the impact of pertussis toxin treatment on [35 S]GTP γ S binding in the absence and presence of muscarinic agonists (not shown).

TABLE 1

Agonist binding and functional properties: CHO cell membranes

The effect of agonists on [35 S]-GTP γ S binding was measured after 10-min incubation in the absence of unlabeled nucleotides or 1 h incubation in the presence of $3 \mu M$ GDP. Average of 2 to 5 experiments in duplicate.

Agonist	pIC_{50}		Control		+ GDP	
	Control	GDP, GTP	pEC_{50}	Efficacy	pEC_{50}	Efficacy
Acetylcholine	5.08 ± 0.10^a	4.38 ± 0.08	5.45 ± 0.19	100%	4.53 ± 0.15	100%
(rac)-Acetyl-(β -methyl)choline	4.74 ± 0.09^a	4.16 ± 0.05	5.16 ± 0.25	81 ± 2	3.82 ± 0.06	78 ± 5
Acetylthiocholine	3.96 ± 0.13	3.55 ± 0.05	4.28 ± 0.30	49 ± 3	3.61 ± 0.22	22 ± 4
Arecoline	5.16 ± 0.11	4.71 ± 0.05	5.97 ± 0.44	52 ± 5	4.69 ± 0.15	25 ± 6
Carbamylcholine	4.17 ± 0.09^a	3.23 ± 0.06	4.67 ± 0.24	93 ± 12	3.65 ± 0.18	81 ± 5
(rac)-Carbamyl-(β -methyl)choline	3.93 ± 0.06	3.51 ± 0.05	4.37 ± 0.27	47 ± 3	3.88 ± 0.15	33 ± 4
McNeil A-343	5.21 ± 0.04	5.01 ± 0.06	5.55 ± 0.3	51 ± 2	4.59 ± 0.23	17 ± 2
Oxotremorine	6.24 ± 0.14	5.85 ± 0.05	6.64 ± 0.21	62 ± 4	5.91 ± 0.25	16 ± 2
Oxotremorine methiodide	5.71 ± 0.09^a	4.89 ± 0.08	6.15 ± 0.15	84 ± 8	5.06 ± 0.17	77 ± 5
Pilocarpine	5.37 ± 0.22	4.97 ± 0.06	5.65 ± 0.53	42 ± 9	5.19 ± 0.30	13 ± 4

[3H]NMS competition curves were obtained as explained under *Experimental Procedures*, and the IC_{50} values were corrected using the Cheng and Prusoff (1973) equation.

^a These competition curves were shallow, and best-fitted with a two sites model: Table 2.

In the first case, I measured the maximal effect of agonists on [³⁵S]GTPγS binding after 1 h incubation in the presence of large GTP (3 μM) concentrations. The results are summarized in Table 3 and Fig. 2, B and C. The partial agonists' efficacy profile in the presence of GTP was very similar to their profile in intact cells (Fig. 2C), not with their profile in the absence of nucleotides (Fig. 2B). In the presence of GDP (Fig. 3, D-F), an intermediate efficacy profile was observed. It was reasonably correlated with their efficacies in intact cells (Fig. 2E) as well as their efficacies in membranes in the absence (Fig. 2D) or presence (Fig. 2F) of GTP.

In the second case, the potency (pEC₅₀) of acetylcholine, carbamylcholine, and oxotremorine-M for [³⁵S]GTPγS binding stimulation increased significantly after pertussis toxin treatment. In addition, all partial agonists achieved at least 80% of the maximal effect of acetylcholine in toxin-treated cell membranes. These results suggested that, because of the very low G protein density available to muscarinic receptors after pertussis toxin treatment, activation of a few of the muscarinic receptors was sufficient to rapidly saturate the few residual G proteins by GTPγS (i.e., that pertussis-toxin treated membranes possessed spare receptors). I compared acetylcholine dose effect curves at different receptor concentrations (Table 4) to verify this hypothesis. It was necessary to inactivate more than 60% of the muscarinic binding sites by 4-DAMP mustard before decreasing the maximal acetylcholine response (Table 4). The partial agonists' efficacies were identical in membranes from control cells and from cells pretreated with both pertussis toxin and 4-DAMP mustard (not shown). Pertussis toxin treatment decreased the density of G proteins responding to muscarinic receptor activation, but did not affect the agonists' efficacy profile (not shown).

TABLE 2

Full agonist binding properties: CHO cell membranes

[³H]NMS-agonist competition curves were obtained as explained under *Experimental Procedures* after 10-min incubation in the absence of unlabeled nucleotides. A two-sites binding model gave significantly better fits of the competition curves for these agonists. The individual K_D values were calculated using the Cheng-Prusoff (1973) equation. Average of five experiments in duplicate.

Agonist	% High Affinity Sites	High Affinity (pK _H)	Low Affinity (pK _L)
Acetylcholine	31 ± 9%	6.20 ± 0.40	4.58 ± 0.22
(rac)-Acetyl-(β-methyl)choline	21 ± 3%	5.84 ± 0.19	4.24 ± 0.20
Carbamylcholine	25 ± 7%	5.72 ± 0.60	3.65 ± 0.13
Oxotremorine methiodide	20 ± 2%	7.79 ± 0.56	5.19 ± 0.11

TABLE 3

Agonist binding and functional properties in intact cells, and efficacy of [³⁵S]GTPγS binding stimulation in CHO cell membranes in the presence of 1 μM GTP

The agonists affinity (corrected IC₅₀ values) in intact cells and their potency (EC₅₀) and efficiency (E_{max} as a percentage of acetylcholine's) for activation of inositol phosphates accumulation were evaluated by nonlinear curve fitting. Average of three to five experiments in duplicate.

Agonist	pIC ₅₀	pEC ₅₀	Efficacy: Intact Cells	Mem-branes, + GTP ^a
Acetylcholine	4.60 ± 0.40	5.05 ± 0.24	100%	(100%)
(rac)-Acetyl-(β-methyl)choline	4.18 ± 0.13	4.41 ± 0.30	99 ± 14	(87 ± 6)
Acetylthiocholine	3.59 ± 0.1	3.21 ± 0.16	54 ± 10	(N.D.)
Arecoline	4.52 ± 0.08	4.46 ± 0.15	59 ± 12	(45 ± 4)
Carbamylcholine	4.01 ± 0.12	4.73 ± 0.15	103 ± 3	(91 ± 8)
(rac)-Carbamyl-(β-methyl)choline	3.49 ± 0.15	3.62 ± 0.25	84 ± 14	(70 ± 10)
McNeil A-343	5.05 ± 0.08	4.76 ± 0.15	28 ± 6	(15 ± 4)
Oxotremorine	5.78 ± 0.08	5.70 ± 0.15	51 ± 9	(45 ± 9)
Oxotremorine methiodide	5.11 ± 0.15	5.76 ± 0.07	100 ± 2	(93 ± 6)
Pilocarpine	4.13 ± 0.06	3.74 ± 0.15	31 ± 7	(47 ± 4)

^a The agonists' efficacy for [³⁵S]GTPγS binding stimulation was measured in CHO cell membranes, in the presence of 1 μM GTP (two experiments in duplicate).

Taken together, these results suggested that the agonists' efficacy profile at M₁ muscarinic receptors depended on the guanyl nucleotides' concentration, but not on the G protein composition.

Discussion

My goal in this work was to test the hypothesis that muscarinic M₁ receptors exist in only two conformations in equilibrium (R and R*) and that all agonists stabilize (with different efficiencies) the one and only "active" receptor conformation, R*. I assumed in addition (for the sake of simplicity) that once the receptor has been activated, it completes at least one G protein activation cycle before deactivating. This model (Appendix) is much more restrictive than the "allosteric model" analyzed by the groups of Lefkowitz and Kenakin (Samama et al., 1993, and Weiss et al., 1995, respectively): these authors indeed included a parameter describing the effect of agonists on G protein recognition by HR*. If the activated receptor's affinity for the G protein depends on the nature of the agonist, this implies that different agonists induce different active receptor conformations.

I analyze in the Appendix a strictly two-states model of G protein activation. Three parameters were necessary to describe G protein activation in the absence and presence of agonists. First, the constant $J = [R^*] / [R]$ is related to the free energy difference (ΔG°) between the (empty, uncoupled) resting and activated receptor conformations ($J = e^{-(\Delta G^\circ/RT)}$). Second, the allosteric parameter β (defined by $\beta J = [HR^*] / [HR]$) is related to the effect of agonist recognition on the free energies of the resting and activated receptor conformations. Agonists are characterized by β >> 1, neutral antagonists by β = 1, and inverse agonists by β << 1. Third, the variable δ, defined by the equation

$$\delta J = \frac{[R^*] + [R^*G_{GDP}] + [R^*G] + [R^*G_{GTP}]}{[R] + [RG_{GDP}] + [RG] + [RG_{GTP}]},$$

is a measure of the (empty + occupied) G proteins' ability to stabilize the active receptor. In contrast to J and β, δ is not a thermodynamic constant: it depends not only on the G protein subtypes encountered by the receptor but also on the nucleotides (GDP, GTP...) concentrations. Pertussis and cholera toxins (which inactivate G_{i/o} and persistently activate G_s proteins, respectively), guanyl nucleotides (which affect

the G proteins' conformations), and the tissue or cell lines' G protein expression pattern may profoundly affect the value of δ .

The agonists' efficacy is usually defined as the maximal over-basal effect (E_{\max}) induced by the ligand of interest, relative to the maximal over-basal effect of the ligand that induced the largest E_{\max} . I had no information about the extent of receptor activation in the absence of agonist (constitutive receptor activity, δJ), or on the ability of acetylcholine to switch the receptors in the active state (β). I therefore used an unusual scale to describe the agonist effects in Figs. 3 and 4: "0" represented the G protein activation rate by resting receptors (R), and "1" represented G protein activation by fully activated receptors, R^* .

The basal rate of G protein activation in the absence of agonist is proportional to $e_0 = \delta J / (I + \delta J)$, and the G protein activation rate observed in the presence of a saturating li-

gand concentration is proportional to $e_{\text{ligand}} = \delta \beta J / (1 + \delta \beta J)$. As shown in Fig. 3 (dotted and dashed lines), if the proportion of empty but nevertheless activated receptors is very low ($\delta J \approx 0$), the constitutive activity of the receptors will be negligible ($e_0 \approx \delta J$) and the efficacy of most agonists, proportional to their ability to stabilize the active receptor conformation ($e_{\text{ligand}} \approx \delta \beta J$). Very efficient agonists ($\beta \gg 1$) are necessary to fully activate the receptors, and inverse agonists cannot significantly decrease the (already negligible) receptor activity. Should G proteins interact significantly and preferentially with activate receptors (Fig. 3, full line), δJ will become large. The rate of receptor-induced G protein activation in the absence of agonists (e_0) will then become significant, and the effect of inverse agonists will become readily detectable. Because of the cooperation of G proteins in maintaining the receptors in the active state, agonists that have a rather small effect on the active receptor conformation's stability (β near 1) may nevertheless fully activate the receptors (Fig. 3, full line).

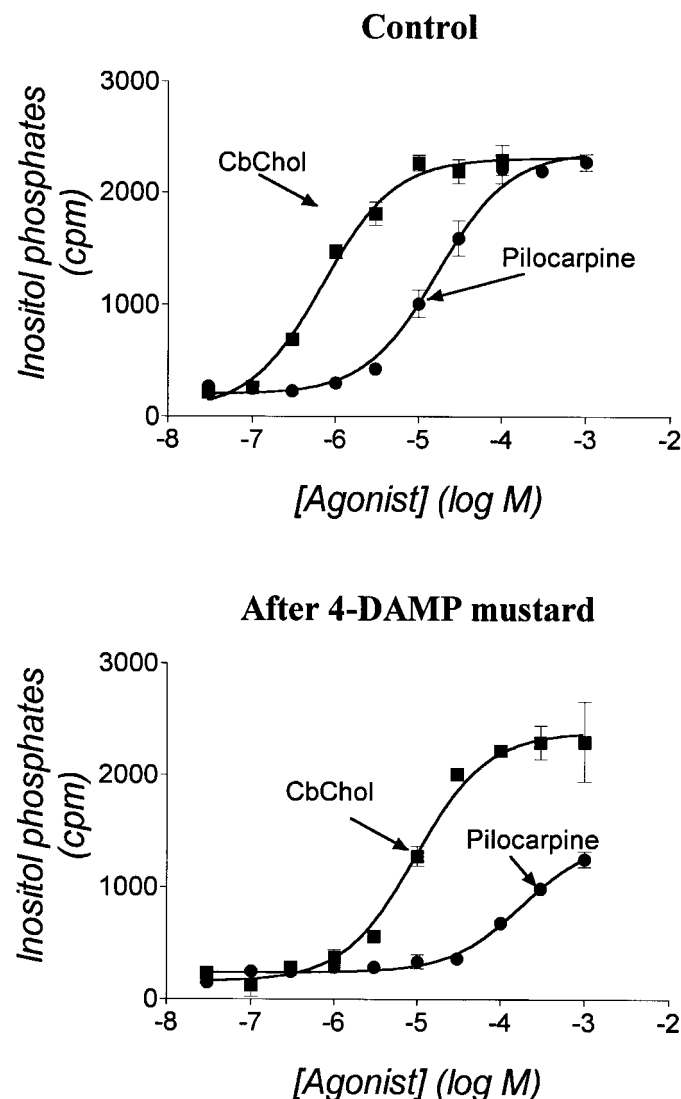


Fig. 1. Presence of spare receptors in the inositol-phosphates synthesis assay. Carbamylcholine (■) and pilocarpine (●) dose-effect curves were obtained in control cells and in cells pretreated for 1 h with 30 nM 4-DAMP mustard before extensive washing. The carbamylcholine and pilocarpine EC_{50} values decreased in this experiment from 6.16 ± 0.07 and 4.80 ± 0.06 to 5.02 ± 0.09 and 3.75 ± 0.08 , respectively. These data are representative of three experiments in duplicate.

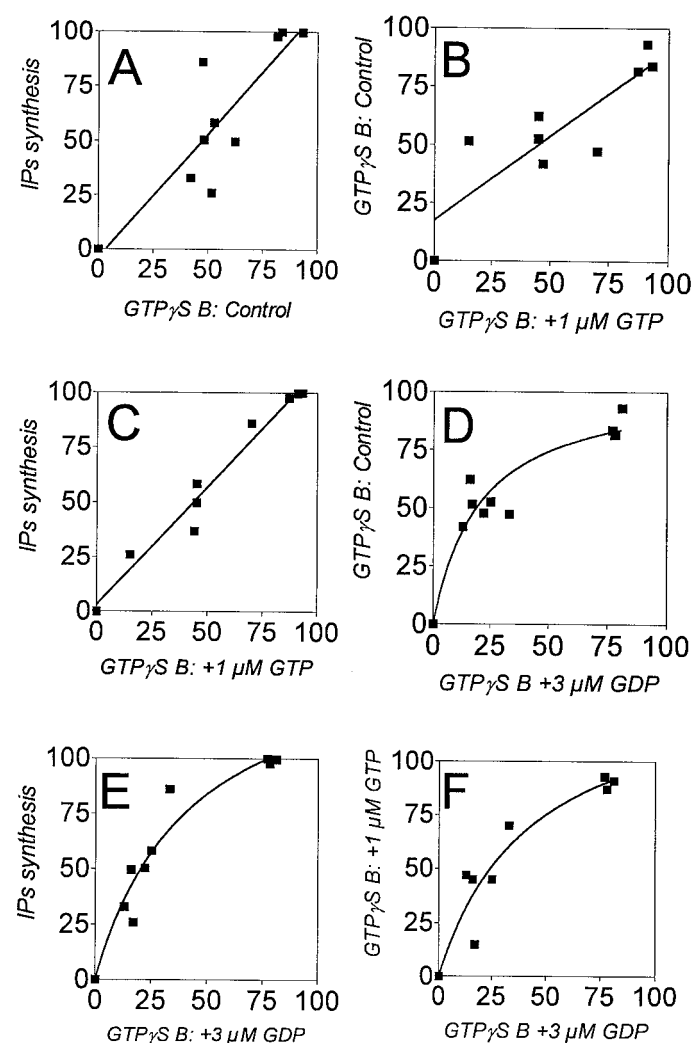


Fig. 2. Comparison of the agonists efficacy profile under different incubation conditions. The efficacy of partial agonists in inositol phosphates accumulation ($n = 4$ experiments, left) and [^{35}S]GTP γ S binding assays ($n = 2$ to 4 experiments, right) are compared with their efficacies for [^{35}S]GTP γ S binding stimulation in the absence ($n = 4$; A) or presence of 1 μM GTP ($n = 2$; B and C) or of 3 μM GDP ($n = 3$; D to F), respectively. Data are from Tables 2 and 3. The lines represent the best fit linear or hyperbolic correlation between the two sets of measurements.

The agonists' potencies (EC_{50} value) do not merely reflect their affinities for either uncoupled (R) or precoupled receptors (RG), but also depends on the G protein's ability to "preactivate" the receptors, δ . Let us assume that there are no spare receptors in the membranes and that the G_{GTP}^* deactivation reaction is efficient (because of rapid GTP hydrolysis), so that the available (empty or GDP-bound) G protein concentration can be considered as approximately constant, even in the presence of agonists. Agonists will then activate all G protein subtypes through the same "receptor

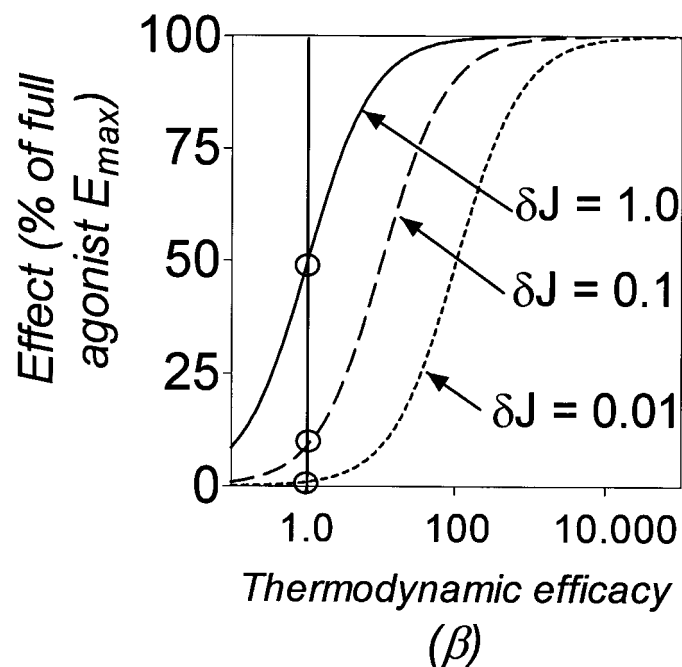


Fig. 3. Two-states allosteric activation. Correlation between the agonist contribution to the active receptor conformation stability and its ability to support G protein activation. The ligand-bound receptor's ability to activate G proteins is plotted on an absolute scale as a function of the ligand's "thermodynamic efficacy" (β), which measures its ability to either stabilize ("agonists", $\beta > 1$) or destabilize ("inverse agonists", $\beta < 1$) the active receptor conformation. The receptor's constitutive activity, measured in the absence of ligand or in the presence of a neutral antagonist ($\beta = 1$), is indicated by an open circle. Three curves are presented: the full line corresponds to conditions in which the empty receptor's interaction with (empty + occupied) G proteins is sufficient to preactivate 50% of the receptors ($\delta J = 1.0$), the dashed line, to conditions in which the receptor-G protein interaction results in only 10% of constitutive receptor activation ($\delta J = 0.1$), and the dotted line, to conditions in which the receptor-G protein interaction is either negligible or insufficient to support significant receptor activation in the absence of agonist ($\delta J \rightarrow 0$).

TABLE 4

Presence of spare receptors in pertussis-toxin treated cells: effect of 4-DAMP mustard on the muscarinic binding site and acetylcholine induced [35 S]GTP γ S binding
Representative of two experiments in duplicate.

4-DAMP mustard	[3 H]NMS (B_{max}) ^a	[3 H]NMS (B_{max})	[35 S]GTP γ S (B_0) ^b
	fmol/mg of protein		% of control
0	760 \pm 29	100	100
10 nM	266 \pm 46*	35 \pm 6*	99 \pm 10
30 nM	100 \pm 30*	13 \pm 4*	42 \pm 3*

^a The muscarinic receptor density (B_{max}) was evaluated by nonlinear curve fitting of [3 H]NMS saturation curves.

^b The difference between tracer (50 pM) [35 S]GTP γ S binding in the absence and presence of acetylcholine (1 mM) was measured in the absence of unlabeled guanyl nucleotides.

* Significantly different from control ($P > 0.95$).

state", with a Hill coefficient (n_H) = 1. Their expected potency (EC_{50}) value will reflect their average affinity for the uncoupled + coupled receptors present in the membrane, somewhere between K_H (their high affinity for the ternary complex) and K_L (their low affinity for uncoupled receptors) (Fig. 4).

Nucleotides may affect the potency and efficacy of GPCR-G protein coupling (Meller et al., 1992; Spengler et al., 1993;

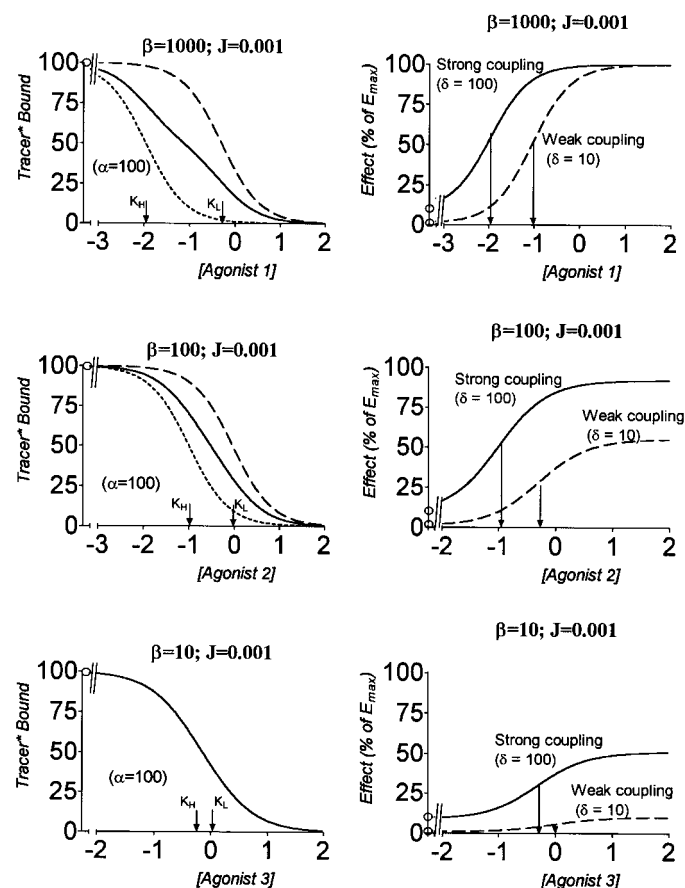


Fig. 4. "Two-states" allosteric activation. Comparison of theoretical agonist competition curves in the absence of guanyl nucleotides with their predicted dose-effect curves. Left, three agonist competition curves (in the absence of guanyl nucleotide) were calculated under the following assumptions: 1) the tracer is a neutral antagonist; 2) the total G protein concentration represents only 50% of the receptor concentration, and 3) only 0.1% of the uncoupled, empty receptors are activated ($J = 0.001$, and the activation free energy, $\Delta G^\circ \approx 18 \text{ kJ} \cdot \text{mol}^{-1} \cdot ^\circ\text{K}^{-1}$). Empty G proteins recognize and thereby stabilize the active receptor conformation: they decrease the receptor's activation energy by $\approx 12 \text{ kJ} \cdot \text{mol}^{-1} \cdot ^\circ\text{K}^{-1}$. Consequently, 10% of the empty G protein-coupled receptors are activated in the absence of agonist ($\alpha J = [R^*G] / [RG] = 0.1$). The agonists' affinities for G protein-coupled and uncoupled receptors (HRG and HR) are indicated as K_H and K_L , respectively. Right, the same agonists' dose effect curves (in the presence of a GTP analog) were calculated under the assumptions that the G protein concentration and affinity are sufficient to maintain either 10% (full line, $\delta J = 0.1$) or only 1% (dashed line, $\delta J = 0.01$) of the receptors in the active state in the absence of agonist. The effect of increasing agonist concentrations is plotted on an absolute scale, where "0" represents the effect of resting receptors, R (for instance, in the presence of a saturating concentration of very active inverse agonist), and "1", the response expected if all the receptors had been switched to the active conformation, R*. The agonists' contribution to the active receptor conformation's stability (β) decreases from the top to bottom panels. In the top panels: β is 1000, and the agonist contributes approximately $18 \text{ kJ} \cdot \text{mol}^{-1} \cdot ^\circ\text{K}^{-1}$ to the active receptor states' stability; in the center panels, $\beta = 100$ and the agonist contribution $\approx 12 \text{ kJ} \cdot \text{mol}^{-1} \cdot ^\circ\text{K}^{-1}$; in the bottom panels, $\beta = 10$ and the agonist contribution, only $\approx 6 \text{ kJ} \cdot \text{mol}^{-1} \cdot ^\circ\text{K}^{-1}$.

Robb et al., 1994; Berg et al., 1998; Bonhaus et al., 1998; Breivogel et al., 1998; Seifert et al., 1999). GDP, like GTP, markedly inhibited the binding of muscarinic agonists (Tables 1 and 2): they prevented G proteins from stabilizing agonist-bound activated receptors and decreased δJ . I therefore expected guanyl nucleotides to decrease the agonists' efficacies (e_{ago}) and potencies (K_{act}) (Fig. 4). If full and partial agonists differ only in their ability to stabilize the same (one and only) activated receptor conformation, their rank order of efficacies in the absence and presence of nucleotides should remain (nonlinearly) correlated (Fig. 5).

The agonists' efficacies for [35 S]GTP γ S binding stimulation in the absence of unlabeled nucleotides were "on average" equivalent to their efficacies in intact cells or in the presence of GTP (Fig. 2, A and B). The correlation, however, was poor (Fig. 2A): there was a 6.5% chance of randomly observing such a substantial incubation condition effect in an experiment of this size (two-way ANOVA, $n = 4$ experiments). The agonists' efficacies for [35 S]GTP γ S binding stimulation in the presence of GTP were well correlated with their effect on inositol phosphates' synthesis activation (Fig. 2C), and pertussis toxin pretreatment did not affect the agonists' efficacy profile in the absence of spare receptors (not shown). These two results suggested that the guanyl nucleotide (but not G protein) composition of the incubation system was an important determinant of the agonists' efficacy profile.

According to the "Cassel and Selinger", G protein activation cycle [accompanying article (Waelbroeck, 2001)], the rate-limiting step for G protein activation depends on the nucleotide concentration. M_1 muscarinic agonists accelerated

G protein activation in the presence of high GTP concentrations, by facilitating the GDP release, and facilitated binding of [35 S]GTP γ S (at very low concentrations) to empty G proteins by accelerating the $G_{GTP\gamma S}^*$ release. Both effects contribute to facilitated [35 S]GTP γ S binding in the presence of GDP: this probably explains why the agonists' effect in the presence of GDP was reasonably correlated with their effect in both other incubation conditions (Fig. 2, D–F).

Taken together, my results did not support the "two conformations" model of G protein activation. There are two possible interpretations for the discrepancies:

To simplify the equations, I assumed in the "two-states" model (Appendix of this and the accompanying article) that the activated receptor does not deactivate before completing at least one G protein activation cycle. If some agonists are released faster than the activated G protein by the HRG_{GTP}^* complex, part of the ternary complex might become trapped in a nonproductive cycle ($HR^*G + GTP \rightarrow HR^*G_{GTP}^* \rightarrow H + R^*G_{GTP}^* \rightarrow H + RG_{GTP}^* \rightarrow H + RG + GTP \rightarrow HR^*G + GTP$, etc.).

If different agonists stabilize different receptor conformations, the variable δ might depend not only on the G protein and guanyl nucleotide composition of the incubation medium but also on the agonist itself.

In conclusion: my results did not support the two-states model of G protein activation.

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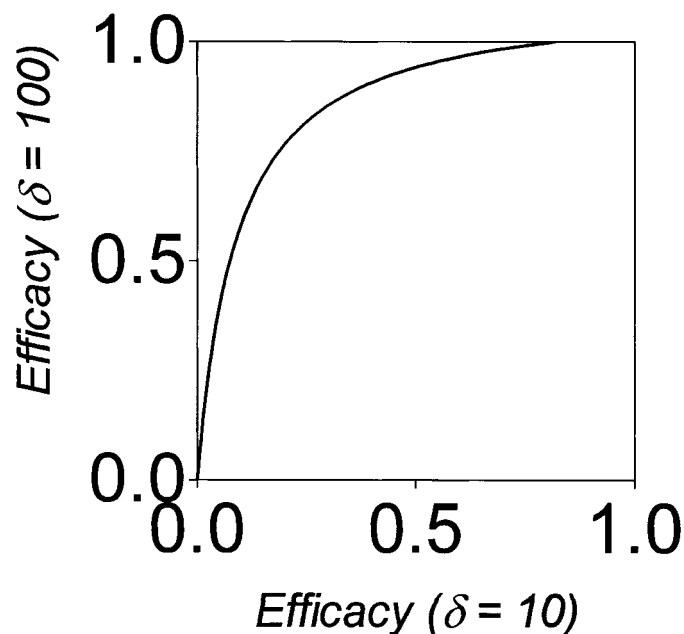


Fig. 5. Two-states allosteric activation. Correlation between the partial agonists' experimental efficacy profiles in weak and strongly coupled systems. The expected efficacy of a set of partial agonists were calculated for two values of " δ ", the variable that describes the G protein's ability to stabilize activated receptors in the absence of agonists (Appendix). G proteins cooperate with agonists for receptor activation: if δ is large, all agonists' efficacies will increase. If the receptors can be found in only two conformations (resting and activated), the rank order of agonists' efficacies will remain independent of the value of δ , and the partial agonists' efficacies, measured under different incubation conditions, should always remain nonlinearly correlated.

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