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Spontaneous Association between Opioid Receptors and GTP-Binding Regulatory Proteins in Native Membranes: Specific Regulation by Antagonists and Sodium Ions

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SUMMARY

High affinity GTPase in membranes from NG108-15 cells was differentially affected by opioid competitive antagonists; one type of antagonist ([*N,N'*-diallyl-Tyr^1-Aib^{2,3}]Leu-enkephalin) reduced the basal rate of GTP hydrolysis, whereas a second type (MR 2266) produced no changes. The inhibitory effect of the "active" antagonist was stereospecifically reversed by the "inactive" antagonist, indicating that it was receptor mediated. This suggests that part of basal GTPase activity in this system results from a spontaneous interaction between opioid receptors and GTP-binding proteins (G proteins) and that some antagonists exhibit negative intrinsic activity by hindering such an interaction. The inhibitory effect of the antagonist was minimal in the presence of Na^+ and maximal when Na^+ was replaced by K^+ in the reaction. When the ratio $[Na^+]/[K^+]$ was progressively increased at constant $[Cl^-]$, total GTPase activity (i.e., net difference between activity stimulated by agonist and that inhibited by antagonist) did not change, but the activity measured in the absence of ligand was selectively decreased. Thus, Na^+ does not alter the total proportion of G proteins that can be activated by ligand-occupied receptors and instead regulates the interaction between receptor and G protein in the absence of ligand. Upon examination of several opioid agonist and antagonists, we found an inverse relation between the intrinsic activity (either negative or positive) of each ligand and the sensitivity to Na^+ of the GTPase elicited upon occupation of the receptor by that ligand. Sodium-mediated and ligand-mediated regulations of GTPase had identical requirements for Mg^{2+} ($[Mg^{2+}]_{free} > 10$ μM), and were both abolished with a similar potency by pertussis toxin. There was no effect of Na^+ on the basal rate of GTP hydrolysis of G_i/G_o purified from bovine brain. However, addition of these proteins to membranes prepared from cells that had been previously exposed to pertussis toxin partially restored both receptor- and sodium-mediated regulations of GTPase in parallel and in a concentration-dependent fashion. We conclude that sodium ions play a key role in the mechanism underlying the spontaneous interaction between "empty" receptors and G proteins in intact membranes.

The properties of the interaction between a receptor and a G protein are best studied in reconstituted systems, where purified components are inserted into phospholipid vesicles and the mechanism of activation can be studied under a well defined set of experimental conditions (see review in Ref. 1). Following this approach, the interactions between β-adrenergic receptors and G_s have been extensively studied (2–5), and the data largely confirm previous information obtained from the kinetic analysis of β-adrenergic receptor-mediated activation of adenylate cyclase in membranes (6, 7). An important outcome of these studies is that receptor appears to activate G protein catalytically, when both are present in synthetic membranes, i.e., a single molecular receptor can promote the activation of several molecules of G protein (2–5). Moreover, both in systems where the three components receptor, G protein and cyclase have been reconstituted (4) and in native membranes (6), the activation of cyclase is a linear function of the concentration of receptor. It has been proposed that for this and perhaps other types of G_s-interacting receptors the ternary complex between agonist, G_s-interacting receptor, and G protein exists only as a transient intermediate during the process of activation (at least under physiological concentrations of GTP), whereas G_s might form a more stable complex with cyclase (8).

A number of reconstitution studies have been also recently

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ABBREVIATIONS: G proteins, family of GTP-binding regulatory proteins, transducing receptor signals; G_s and G_i, GTP-binding proteins mediating stimulation and inhibition of adenylate cyclase, respectively; G_o, GTP-binding protein that is substrate or pertussis toxin found in high concentrations in brain tissue and has less known functions; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; ICI 174864, [*N,N'*-diallyl-Tyr^1,Aib^{2,3}]Leu-enkephalin; ICI 154129, [*N,N'*-diallyl-Tyr^1, ψ (CH$_2$S)-Phe^4]Leu-enkephalin; App(NH)p, adenosine 5′-(2,3-imido)triphosphate; DADLE, [D-Ala^2-D-Leu^5]-enkephalin; DPDPE; [D-Pen^2,D-Pen^5]-enkephalin.

reported for those receptors interacting with G proteins that are substrate of pertussis toxin (9–12), but less detailed information on the properties of this interaction is available. This group of receptors (which includes some muscarinic subtypes, α_2-adrenergic, D_2 dopamine, and opioid receptors) is usually linked in negative manner to adenylate cyclase in native membranes (see review in Ref. 13). A possibly common feature of these receptors is their tendency to form very stable complexes with G proteins even when no agonist is added before solubilization (9, 14). This suggests that the interaction between G_i-interacting receptor and G_i/G_o may involve a much tighter coupling than that between G_s-interacting receptor and G_s. Indirect evidence for such a tight interaction has been obtained in native membranes, where either the analysis of the kinetics of agonist and antagonist binding to α_2-adrenergic receptors (15) or computer modeling of D_2 dopamine equilibrium binding isotherms (16) suggests the existence of precoupled G_i-interacting receptor-G protein complexes. For opioid receptors, we drew similar conclusions from target size analysis of high affinity agonist binding using membranes in which receptors had been uncoupled by pertussis toxin or *N*-ethylmaleimide (17). The examination of the pattern of loss of GTPase responsiveness to opioid receptors following agonist pretreatment (18), receptor alkylation, and G protein inactivation by pertussis toxin (19) also suggested that opioid receptor and G proteins are precoupled in these membranes. If a fraction of the G proteins present in a given membrane can couple to receptors in the absence of agonists, then a fraction of the high affinity GTPase activity measurable in that membrane would reflect this interaction. Spontaneous interactions between "empty" receptor and G protein have been shown to occur in liposomes (3, 20), but a proof for the existence, and the exact characterization, of this phenomenon in native membranes is difficult to achieve, due to the uncertainty regarding the stoichiometry of the components involved. This has seriously limited the interpretation of data obtained from the study of GTPase activity in membranes (21).

However, if part of the basal GTPase activity reflects spontaneous coupling between receptor and G protein, it might be expected that some antagonists would disrupt this interaction and thus inhibit GTPase to some extent, if there are antagonists endowed with negative efficacy, that is, antagonists that upon binding to "empty" receptors can induce a conformation of receptor less able to interact with G protein (16). Based on this criterion, we have recently shown that two classes of opioid antagonists can be distinguished, those with negative and those with null intrinsic activity. The effect of negative antagonists was not due to contamination of membranes with endogenous agonists and required receptor occupancy by the antagonist and an active G protein in the membrane (22). However the effect of the antagonist, unlike that of the agonist (23), was most evident in the absence of Na^+.

In the present study we have examined in detail the ionic requirements for antagonist-mediated inhibition of GTPase in membranes from NG108-15 cells. We show here that sodium ions are specific regulators of the spontaneous interaction between receptor and G protein in the absence of agonist.

Experimental Procedures

Materials. [γ-^{32}P]GTP (6000 Ci/mmol) and [^{35}S]GTPγS (800–1300 Ci/mmol) were obtained from New England Nuclear. ATP (Tris or Na^+), App(NH)p (Li^+), GTP (Tris), phosphocreatine (Tris), phosphoenolpyruvate (K^+ or cyclohexylammonium), pyruvate kinase (540 units/mg, type III), creatine phosphokinase (100–150 units/mg, type I), HEPES, EDTA, EGTA, and cyclohexylammonium sulfate and phosphate were purchased from Sigma. GTPγS and Tris base were from Boehringer-Mannheim. The chloride salt of *N*-methyl-glucamine was prepared from the base (Sigma) in water and purified by crystallization in ethanol/isopropyl alcohol mixtures. Analysis: ($C_7H_{18}NO_6Cl$) C 36.73%, H 8.29%, N 6.01%, Cl 15.3%. Pertussis toxin was purchased from List Biologicals. Lubrol PX and cholic acid (Sigma) were purified as described (24). DADLE and DPDPE were from Bachem, ICI 174864 and 154129 from Cambridge Research Biochemicals. Naloxone and naltrexone were a gift of Dupont de Nemours. MR 2266 and MR 2267 were donated by Dr. Merz (Boehringer, Ingelheim). Diprenorphine was a gift of Reckitt and Colman. All other chemicals were purchased from commercial sources and were of the best purity available.

Membranes and GTPase assay. Culturing of NG108-15 cells and preparation of membranes was done as described previously (18, 19). The differential centrifugation protocol for the isolation of the 25,000 × *g* membrane fraction used here (18) is an important variable for reproducibility, because homogenization of NG108-15 cells produces an additional "light membranes" fraction (pelletted at 200,000 × *g* from the supernatant of 25,000 × *g* membranes) in which high levels of low K_m GTPase activity are present but agonist-stimulated and antagonist-inhibited activities are barely detectable (25). The GTPase reaction mixture for the experiments described here either was identical to that reported previously (18, 19) (referred to as Buffer A) or was modified to study the effect of ions. The modified reaction mixture (referred to as Buffer B) included 0.2 mM EGTA, 0.2 mM dithiothreitol, 0.5 mM ATP (Tris), 1 mM App(NH)p (Li^+), 5 mM phosphocreatine (Tris), 50 units/ml creatine phosphokinase, 10 mM $MgSO_4$, 50 mM HEPES/Tris (pH 7.5), and 2.5 mM cyclohexylammonium phosphate. NaCl or KCl or a mixture of these two salts was present at concentrations indicated in each experiment; when not stated otherwise, the total ion concentration used was 150 mM. [γ-^{32}P]GTP was used at a final concentration of 200 nM and final specific activities between 30,000 and 100,000 cpm/pmol, depending on the requirements of each experiment. In some experiments the ATP-regeneration system was replaced by 5 mM phosphoenolpyruvate and 5.4 units/ml pyruvate kinase. When the effect of Mg^{2+} was studied, the reaction included a fixed concentration of 1 mM EDTA and varying concentrations of $MgSO_4$; pH did not change over the entire range of these metal/EDTA concentrations employed. Purified G proteins were assayed in a similar reaction mixture except for the omission of the regeneration system. Reactions were started by the addition of membranes (or G proteins), conducted at 37° for 10 min (except when stated otherwise) in a total volume of 0.1 ml, and arrested by the addition of ice-cold 40 mM H_3PO_4 (pH 2). Tubes were rapidly transferred to ice and 0.75 ml of ice-cold 5% (w/v) charcoal in 20 mM H_3PO_4 was then added to each tube. Determination of the $^{32}P_i$ released was done as described (18). The high K_m nucleotidase activity of the membranes was subtracted from the data, as described by Cassel and Selinger (21), and was determined in each experiment by measuring the extent of hydrolysis observed at 50 μM GTP. This activity was also altered by ionic changes in the reaction mixture. For example, substitution of K^+ by Na^+ at a constant ionic strength produced 20–30% stimulation of this enzymatic activity, an effect that could be attributed to inhibition by K^+ rather than to stimulation by Na^+. Detailed curves for P_i release as a function of GTP concentration obtained in 150 mM levels of either KCl and NaCl indicated that the position of the plateau of hydrolysis observed at high GTP concentrations (10–100 μM) on the abscissa was not altered by Na^+. Thus, the hydrolysis observed at 50 μM GTP remains a valid estimate of the contribution of low affinity activity under either ionic condition. At the concentration of substrate used in these experiments (200 nM), the high affinity component of hydrolysis accounted for 60–75% of the total P_i released. Nonenzymatic (background) hydrolysis of GTP varied

with the quality of the radiolabeled nucleotide and accounted for 0.5–1.8% of the total cpm measured.

Purification of G proteins. Purified preparations of pertussis toxin substrates from bovine brains containing varying proportions of G_i/G_o were obtained according to the method of Sternweis and Robishaw (26), with minor modifications as described previously (27). The preparation used in these experiments contained approximately equal proportions of G_i and G_o, as determined by silver staining of polyacrylamide gels, Ferridye staining of nitrocellulose blots, and Western blot analysis with a pan-reactive antibody. This corresponded to a discrete peak of GTPγS-binding activity eluted from the octyl-Sepharose column, the characterization of which has been described previously (see peak 3 in Ref. 27). The stock solutions of G_i/G_o were stored at a protein concentration of 1.6 mg/ml in small aliquots at −80°, in a buffer of the following composition: 1% sodium cholate, 100 mM NaCl, 25 mM Tris·HCl, pH 8, 1 mM dithiothreitol, and 1 mM EDTA. The molar concentration of active proteins was determined by saturation analysis with [^{35}S]GTPγS, using an incubation mixture similar to that used for GTPase (Buffer B, but without adenine nucleotides and regeneration system) and a reaction time of 90 min at 20°. Determinations of bound/free ratio were done by filtration on BA-85 filters (Schleicher and Schuell) as described (26). Saturation isotherms were consistent with a single class of binding sites and a maximal binding of 0.6 mol/mol of protein. This value was used to estimate the pmol of G protein employed in each experiment. The turnover number for GTPase (estimated from Scatchard plots) of this preparation was 0.29 mol^{-1}. Pertussis toxin-catalyzed ADP-ribosylation of purified G proteins was done in a reaction mixture that included 0.1% Lubrol PX (w/v), 10 mM dithiothreitol, 1 mM NAD^+, 10 μM ATP, 50 mM HEPES/Tris (pH 7.5), 20 μg/ml pertussis toxin, and 3–6 pmol of G protein; reactions were conducted in a total volume of 10 μl, at 30° for 10 min. Control reactions contained an identical amount of G protein but, instead of toxin, its diluent (50 mM Tris·HCl, pH 7, 100 mM NaCl). Reactions were arrested by dilution (at least 16-fold) with ice-cold 5 mM HEPES/Tris, 1 mM EDTA, 1 mM dithiothreitol (pH 7.5) containing 0.1% bovine serum albumin, and the ribosylated proteins were immediately used for GTPase assays or reconstitution experiments. Even with the highest concentration of G protein employed, the components of the ribosylation reaction mixture were diluted 160-fold in the final GTPase reaction. The effectiveness of ADP-ribose incorporation was qualitatively evaluated in Western blots by the reduction of R_f of the immunoreactive bands corresponding to G_i and G_o (28). Completeness of ADP-ribosylation is indicated by the inability of modified G protein to reconstitute receptor-mediated stimulation.

Reconstitution of receptor-dependent GTPase activity in membranes of cells treated with pertussis toxin. To monitor reconstitution of agonist- and antagonist-mediated regulation of GTPase in membranes prepared from intoxicated cells, we used a protocol similar to that described by Milligan and Klee (29). Membranes from cells treated or not (controls) with pertussis toxin (10 ng/ml, 24 hr) were premixed with different amounts of G protein (as indicated in each experiment) at 37° for 2 min, in a total volume of 50 μl of a buffer consisting of 5 mM HEPES/Tris (pH 7.5 at 37°), 0.4 mM levels of both EGTA and dithiothreitol, and 0.1% (w/v) bovine serum albumin, in the presence or absence of opioid ligands. Reactions were started by the addition of prewarmed 2-fold concentrated Buffer B (without EGTA and dithiothreitol) containing [γ-^{32}P]GTP (200 nM final concentration, 80,000–100,000 cpm/pmol), conducted for 15 min at 37°, and arrested as described for the GTPase assay in membranes. Nonspecific hydrolysis of GTP was subtracted using assays containing 50 μM GTP, as described above. The contribution of cholate in the final assay mixture by the highest concentration of G proteins employed was less than 0.006% (w/v). G proteins that had been denatured by heat (95°, 5 min) or ADP-ribosylated by pertussis toxin (see Purification of G Proteins) were used as controls in these reconstitution experiments.

Calculation of the free Mg^{2+} concentration. In some experiments the total concentration of Mg^{2+} was varied in the presence of a fixed concentration (1 mM) of EDTA. Estimates of the concentration of the free ion are based on the assumption that the metal exists either in nonbound form or complexed to the chelating agents and nucleotides present in the reaction. Free Mg^{2+} was computed by solving simultaneously the following set of equations:

$$B/F = \sum_{j=1}^{n} [K_{1_j} S_j/(1 + K_{1_j} F)]$$

$$T = F + \sum_{j=1}^{n} B_j$$

where T, B, and F are $[Mg^{2+}]_{total}$, $[Mg^{2+}]_{bound}$, and $[Mg^{2+}]_{free}$, respectively; S_j is the total concentration of each complexing species j (e.g., EDTA, ATP, GTP, etc.) present in the reaction; K_{1_j} is the affinity constant of the metal for each species; and B_j is $[Mg^{2+}]_{bound}$ to each species. Numerical solutions were obtained by the iterating procedure of Feldman *et al.* (30), using a subroutine written in the Basic computer language by Munson and Rodbard (31). The following affinity constants of Mg^{2+} for chelating agents and nucleotides (32) were used (in M^{-1}): EDTA, 10^6, ATP and App(NH)p (assumed to be identical), 1.74×10^4; GTP, 6.4×10^4; EGTA, 7.3; and phosphocreatine, 20. The validity of the calculations was checked, in some cases, by repeating experiments with 0.2 mM EDTA and a corresponding varying range of smaller total Mg^{2+} concentrations. The position of the peak of Mg^{2+} stimulation varied less than ⅓ of a log unit under these conditions, a variation comparable to that observed between experiments performed with identical EDTA concentrations.

Data analysis and expression of results. The experiments presented here were repeated several times but at least twice and in at least two different batches of membranes. Most of the experiments presented are representative, except where stated otherwise in the corresponding legends. Absolute GTPase activities varied as much as 2-fold from one membrane preparation to another, and so did the net receptor-dependent activity, but the relative proportions of agonist and antagonist effect on GTPase were more constant. GTPase determinations were done in triplicates or in quadruplicates, with a standard error that was between 1 and 3% of the mean cpm values. When net activities were examined, their standard error was computed as the square root of the sum of the variances corresponding to the two means used for the subtraction. The following terminology is used to indicate net activities: total receptor-dependent activity, GTPase in the presence of agonist minus that in the presence of antagonist; net agonist-dependent, that in the presence of agonist minus basal; and net antagonist-dependent, basal minus that in the presence of antagonist.

Results

Specificity of antagonist-mediated inhibition of GTPase in NG108-15 cell membranes. Upon examination of a number of opioid antagonists for their effect on basal high affinity GTPase activity in NG108-15 cell membranes, we noticed that the peptidergic antagonist ICI 174864 produced a consistent inhibitory effect. The degree of inhibition was extremely small (Fig. 1, *top left*) and the effect was difficult to characterize. However, when the usual ATP-regenerating system employed in the reaction (phosphocreatine/creatine phosphokinase) was substituted by a phosphoenolpyruvate/pyruvate kinase system, the inhibitory effect of ICI 174864 became more prominent (Fig. 1 *top right*). This effect of the regeneration system was not due to different abilities in maintaining ATP concentrations (a 10-fold increase in the concentration of the phosphocreatine/creatine phosphokinase system or its total removal had no effect), nor was it due to enzymatic contaminants of the pyruvate kinase system (heat denaturation of the enzyme did not suppress its ability to enhance antagonist

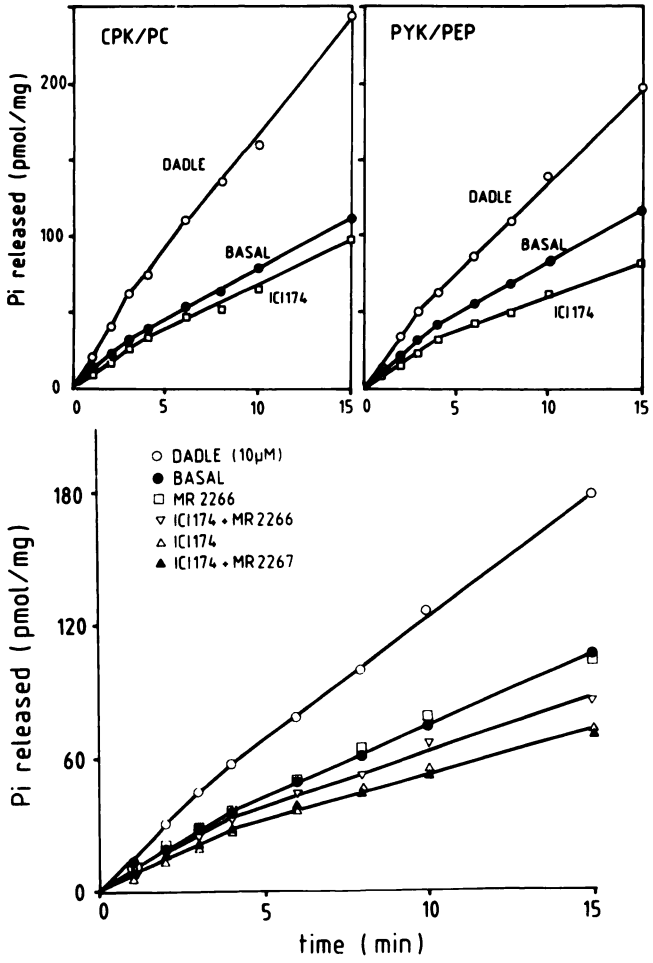

Fig. 1. Effect of opioid antagonists on the basal rate of GTP hydrolysis in membranes. *Top*, Membranes (5.8 μg of protein) prepared from NG108-15 cells were assayed for GTPase in Buffer A containing two different ATP-regeneration systems, creatine phosphokinase/phosphocreatine (*CPK/PC, left*) or pyruvate kinase/phosphoenolpyruvate cyclohexylammonium (*PYK/PEP, right*). DADLE and ICI 174864 were present at 10 μM. *Lower*, Membranes were assayed in Buffer A with creatine phosphokinase/phosphocreatine as the regeneration system and the additional presence of 2.5 mM of cyclohexylammonium phosphate. All ligands were present at 10 μM. *Points* are means of triplicate determinations. The experiments are representative of two (*top*) and three (*bottom*) additional experiments, which yielded similar results.

effect), but it was entirely explained by the counter ion (cyclohexylammonium) of the phosphoenolpyruvate preparation used. The addition of cyclohexylammonium (either phosphate or sulfate) in the presence or absence of phosphocreatine/creatine phosphokinase was sufficient to produce enhancement of antagonist-mediated inhibition. Optimal concentrations were between 2.5 and 5 mM, because at 0.5 mM there was no detectable effect whereas from 20 to 100 mM the ion produced strong inhibition of total activity (data not shown). Although the mechanism of this effect is not clear, we included cyclohexylammonium as a component of the reaction in order to study the ability of ICI 174864 to inhibit basal GTPase in more detail. As shown in Fig. 1, *bottom*, the inhibitory effect of ICI 174864 was receptor specific. A benzomorphan opioid antagonist, MR 2266, did not influence basal GTPase, but it did attenuate the inhibitory effect of ICI 174864 when added in equimolar concentrations. Its (+)-enantiomer, MR 2267, which has little or no affinity for opioid receptor, did not produce any effect on antagonist-inhibited activity (Fig. 1, *bottom*). Although the time-course of GTP hydrolysis in these membranes was not perfectly linear and exhibited two apparent components of faster (0–3 min) and slower (4–15 min) rates, both agonist-mediated stimulation and antagonist-mediated inhibition occurred with no lag phase and without altering the biphasic pattern of the reaction.

A number of experiments were performed to evaluate the specificity of the inhibitory effect the of antagonist. Bradykinin stimulates low K_m GTPase activity in NG108-15 membranes and this stimulation is additive to that of opioid agonists (33, 34). We found that the effect of bradykinin (1 μM) was still detectable in the presence of a maximal concentration of ICI 174864 (data not shown), indicating that the antagonist inhibits only GTPase activity resulting from the interaction of G proteins with opioid receptors. In membranes prepared from cells that had been cultured in serum-free medium, the effect of the antagonist was comparable to that observed in cells grown under routine conditions. When these cells were exposed to pertussis toxin (24 hr, 10 ng/ml), the effect of the antagonist on GTPase was abolished and the basal activity was 40–50% lower than in controls, as observed for cells grown in serum. This suggests that the activity in membranes from NG108-15 cells is not under the constitutive stimulation of a growth factor receptor.

Collectively, these data clearly indicate that the inhibitory effect of ICI 174864 is stereospecifically mediated by opioid receptors and suggest that a certain portion of G proteins are present in these membranes in the form of a preactivated receptor-coupled species. Because the relationship between agonist-stimulated, antagonist-inhibited, and basal activity can be altered by ionic manipulations such as the addition of cyclohexylammonium, the degree of precoupling may be modulated by ions. This possibility, therefore, was investigated further.

Effect of monovalent ions on agonist and antagonist modulation of GTPase. GTPase in membranes is normally assayed in the presence of millimolar concentrations of both NaCl and $MgCl_2$ (e.g., as in our Buffer A) because, as shown by Koski *et al.* (23), both salts are required for the observation of opioid agonist-mediated stimulation, a requirement shared by other receptor types (35). In initial experiments designed to assess the role of these salts in the effect of both agonists and antagonists, we found that antagonist-mediated inhibition was already evident in the presence of $MgCl_2$ and absence of NaCl (data not shown). Because the effects of ions on GTPase may result from multiple and counteracting influences of cations, anions (36), and changes in ionic strength (23), GTPase in the presence of agonist or antagonist or in the absence of ligand was compared under a constant concentration of Mg_2SO_4 and varying concentrations of NaCl, KCl, or *N*-methyl-glucamine chloride (as a control for ionic strength). At low concentrations, both NaCl (Fig. 2a) and KCl (Fig. 2b) affected the activity in the presence of agonist differently than that in the presence of antagonists, inasmuch as the former was slightly stimulated, whereas the latter was sharply decreased. At higher concentrations, both activities declined linearly in parallel. With *N*-methyl-glucamine, only little, if any, reciprocal effect on agonist- and antagonist-dependent activities was evident at low concentrations, and both activities decreased in parallel (Fig. 2c) with slopes similar to those observed for high concentra-

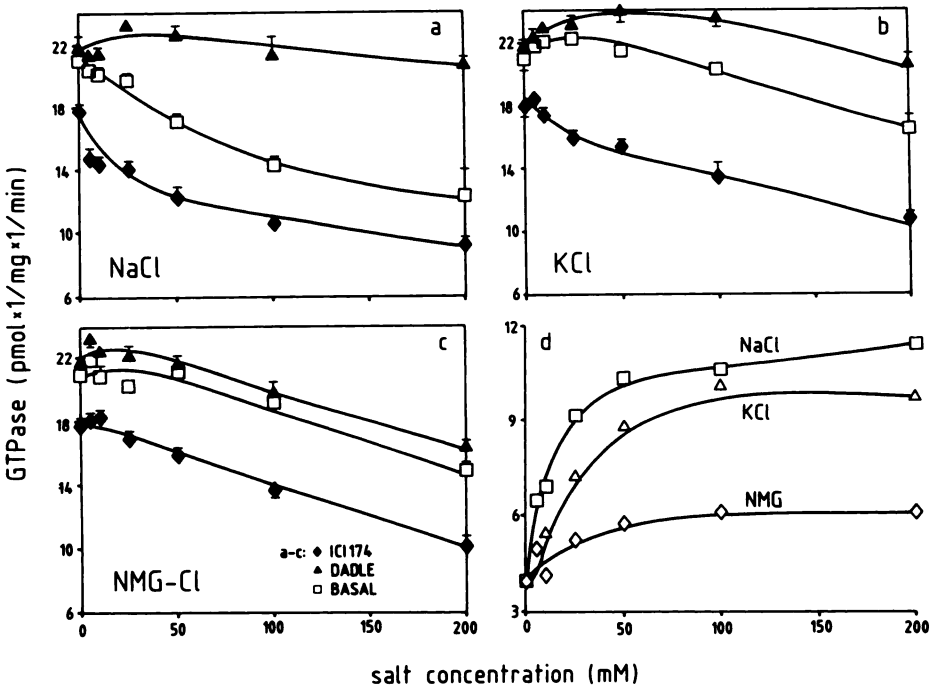

Fig. 2. Effect of ions on GTPase activity measured in the presence of agonist and antagonist. GTPase in membranes from NG108-15 cells was assayed in Buffer B, in the presence of increasing concentrations of NaCl (a), KCl (b), or *N*-methyl-D-glucamine chloride (*NMG-Cl*) (c). The activity in the absence (basal) or presence of 10 μM DADLE or 10 μM ICI 174864 is shown (*symbols* are in c). Data are means ± standard errors of triplicate determinations. d, Net receptor-dependent activity [GTPase in the presence of agonist (DADLE) minus that in the presence of antagonist (ICI 174)] as a function of the concentration for the three salts, as indicated.

tions of KCl and NaCl. The linear component of decrease (similar for all three salts) is, conceivably, a nonspecific effect of ionic strength and can be subtracted when "net" receptor-dependent GTPase (i.e., activity in the presence of agonist minus that in the presence of antagonist) as a function of salt concentration is compared for the three ions (Fig. 2d). This plot shows that both KCl and NaCl produced a stimulation of receptor-dependent activity, whereas *N*-methyl-glucamine chloride was much less effective. There was no clear sodium specificity for this effect. However, Na^+ and K^+ exerted clearly divergent effects on basal activity, inasmuch as at low concentrations basal activity was inhibited by Na^+ but not changed or slightly stimulated by K^+ (compare Fig. 2, and and b). Thus, there are at least two specific effects of cations on GTPase in membranes; one is the increase in the net difference of activity in the presence of agonist and antagonist, for which K^+ and Na^+ but not *N*-methyl-glucamine are similarly effective. The second effect is the inhibition of basal activity, which exhibits a clear Na^+ selectivity. Fig. 3 shows a comparison of the effect of several cations on GTPase (at a constant concentration of 150 mM Cl^- and 10 mM Mg_2SO_4). Basal activity was highest in K^+ and lowest in Na^+ and was inhibited with a rank order of potency ($Na^+ > Li^+ > Rb^+ > K^+$) that was reminiscent of that described for the suppression of high affinity opioid agonist binding.

Effect of Na^+ on agonist-mediated activation and antagonist-mediated inhibition of GTPase activity. We examined GTPase in the presence of agonist and antagonist, using reaction mixtures in which Cl^- and ionic strength were held constant but K^+ was gradually replaced by equimolar concentrations of Na^+ (Fig. 4). As the ratio Na^+/K^+ progressively increased, basal activity was reduced to a larger extent than those in the presence of either agonist or antagonist, whereas their net difference was not significantly changed (Fig. 4, *inset*).

The fact that basal GTPase is more sensitive to Na^+ than

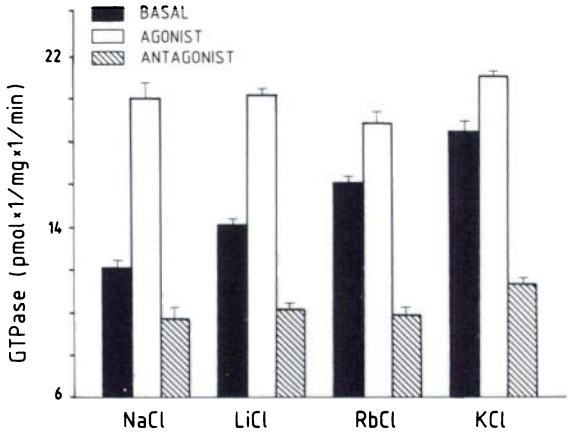

Fig. 3. Effect of different ions on GTPase in the absence or presence of agonist or antagonist. GTPase was assayed in Buffer B in the presence of different salts (all 150 mM) as indicated. Agonist (DADLE) or antagonist (ICI 174864) were present at 10 μM. Each *bar* is the mean ± standard error of quadruplicate determinations. The experiment was repeated twice with identical results.

the activity in the presence of ligand (either agonist or antagonist) suggests that the mechanism responsible for sodium-sensitive basal activity may differ qualitatively from that underlying activation or deactivation by ligand-bound receptor. This is documented in Fig. 5a with data obtained using several opioid agonists and antagonists at their maximally effective concentrations. The decrease of GTPase activity (normalized as percentage of that measured in the absence of ligand and presence of only K^+) is displayed as a function of the logarithm of $[Na^+]/[K^+]$ ratio. In these coordinates, the decrease of activity can be approximated by linear relation, the negative slope of which is a measure of the sensitivity to Na^+. The curves obtained in the presence of ligand are above (agonists), below (antagonist with negative intrinsic activity), or not significantly different (antagonists with null intrinsic activity) from the curve obtained in the absence of ligand (basal) (Fig. 5a).

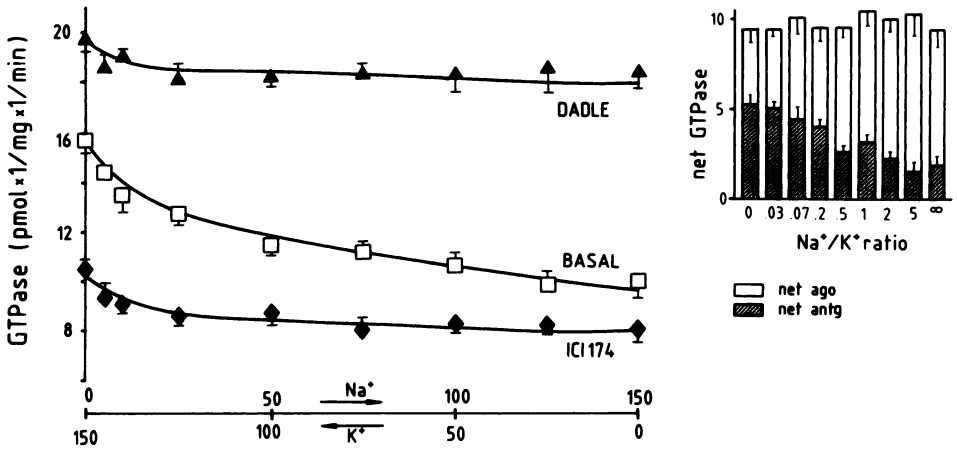

Fig. 4. Effect of substitution of Na^+ with equimolar concentrations of K^+ on GTPase in the presence of agonist or antagonist. GTPase was assayed in Buffer B in the presence of NaCl or KCl mixed in different proportions to give the concentrations indicated on the abscissa while maintaining a fixed total salt concentration of 150 mM. Agonist (DADLE) and antagonist (ICI 174864) were present at 10 μM. Data are means of three different experiments performed (in triplicate) in the same membrane preparation. Similar data were observed using three additional batches of membranes. *Inset*, the data of the main panel are expressed as net activities and replotted as a function of the Na^+/K^+ ratio. Note that the net effect of the antagonist (*antg*) (basal activity minus that in the presence of ICI 174864) and of the agonist (*ago*) (activity in the presence of DADLE minus basal activity) are of comparable magnitude in the absence of any sodium, whereas the former is barely detectable in the presence of only sodium.

Two points emerge from these experiments. First, antagonists exhibited a broad spectrum of differences in their ability to inhibit GTPase; the larger inhibition was observed with the peptide ICI 174864, followed by its close analogue ICI 154129. Only a small effect was observed for naloxone, whereas naltrexone and MR 2266 had little effect on basal GTPase. Thus, antagonists can be classified as "full negative," "partial negative," or "neutral" on the basis of their negative efficacy.

Second, the Na^+ sensitivity of GTPase in the presence of a given ligand was correlated with the intrinsic activity of the ligand. In fact, basal activity and that in the presence of partial agonists or antagonists were inhibited by Na^+ with a steeper slope than that in the presence of full agonists or antagonists. The slopes were progressively reduced the more intrinsic activity (negative or positive) differed from zero. For example compare the agonists diprenorphine, morphine, and DADLE or DPDPE and compare the antagonists naloxone, ICI 154129, and ICI 174864 (Fig. 5a). Such a relation between relative intrinsic activity of opioid ligands and slope of the sodium effect is shown in Fig. 5b. Accordingly, differences in intrinsic activities were most evident at low Na^+/K^+ ratios for antagonists and at high Na^+/K^+ ratios for agonists.

Effect of Mg^{2+} on the sensitivity of GTPase to Na^+. GTPase in the presence of either agonist or antagonist or in their absence was studied as a function of free Mg^{2+} (SO_4 salt) under a fixed concentration of either KCl and NaCl (Fig. 6). There was very little hydrolysis in the membranes in the absence of added Mg^{2+} and presence of 5 mM EDTA. However, as the concentration of free Mg^{2+} was increased, the low K_m activity was sharply stimulated, reaching a maximum at about 1 μM $[Mg^{2+}]_{free}$ and with an apparent EC_{50} for the metal of 60–70 nM. Within this range of Mg^{2+} concentrations, there was no difference in activity between agonist and antagonist or KCl and NaCl. As the concentration of free Mg^{2+} increased from 10 μM to 5 mM, GTPase activity was inhibited but to a different extent depending whether agonist or antagonist and K^+ or Na^+ were present, i.e., both receptor- and cation-mediated regulation of GTPase became evident. Therefore, two types of activities can be distinguished. One requires submicromolar concentrations of Mg^{2+} and it is both Na^+ and receptor independent. Another requires submillimolar concentrations of the metal and it is both sodium and receptor regulated (Fig. 6).

Effect of pertussis toxin treatment of membranes on ionic regulation of GTPase. To examine how pertussis toxin affects the two components of Mg^{2+}-dependent activity described in Fig. 6, we compared basal GTPase (measured in the presence of either K^+ or Na^+) in membranes prepared from cells that had been treated with pertussis toxin (10 ng/ml, 18 hr) or its vehicle, under a wide range of $[Mg^{2+}]_{free}$. Fig. 7a shows that the Mg^{2+}-dependence of GTPase in toxin-treated membranes was qualitatively identical to that observed in control membranes. However, the net difference in activity between control and toxin-treated membranes was also Mg^{2+} dependent. At nanomolar Mg^{2+} concentrations there was no difference in activity between the two membranes. However, as the metal was increased from 10 nM to 1 μM, a difference in rate of hydrolysis between the two membranes became evident; GTPase activity was only 10–18% lower in toxin-treated membranes within this range of Mg^{2+} concentrations and there was no difference in activity when K^+ was replaced by Na^+ (Fig. 7a). At concentrations of Mg^{2+} between 10 μM and 10 mM, the reduction of GTPase in toxin-treated membranes compared with control became progressively larger, and the difference in basal activity between K^+ and Na^+ was clearly detectable in control but much less evident in toxin-treated membranes. This complex Mg^{2+} dependency of the effect of pertussis toxin on basal activity is illustrated in Fig. 7b, where we have plotted the net "toxin effect" (difference of activity between control and toxin-treated membranes, measured in either K^+ or Na^+) together with the net "sodium effect" (GTPase in the presence of Na^+ minus that in the presence of K^+ measured in the two membranes) as a function of $[Mg^{2+}]$. It is clear that the toxin effect exhibited two components. The first component accounted for a smaller portion of the total inhibited activity, occurred at 10 nM to 1 μM Mg^{2+}, and was identical in K^+ or Na^+; conceivably, this reduction of activity reflects an intrinsic diminished responsiveness of ribosylated G proteins to Mg^{2+}. The second component occurred between 10 μM and 10 mM

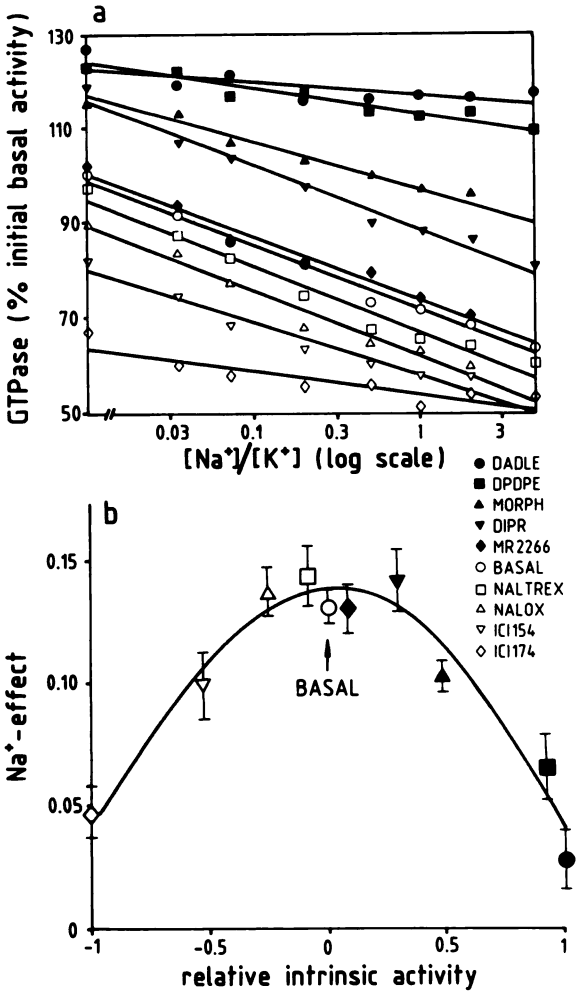

Fig. 5. Relationship between relative intrinsic activity of opioid ligands and the sensitivity to Na^+ of the GTPase elicited upon occupation of the receptor. a, GTPase was assayed as indicated in the legend to Fig. 4. The activity measured in the presence of each ligand is plotted as a function of the logarithm of the Na^+/K^+ ratio. All ligands were present at 10 μM except morphine (*MORPH*) (100 μM) and diprenorphine (*DIPR*) (1 μM). DPDPE is a δ-selective peptide. Concentration-response curves for these ligands obtained in either only KCl or only NaCl indicated that the concentrations used were maximal for their effect on GTPase under both ionic conditions. Data were normalized as percentage of the activity measured in the absence of ligand (basal) and presence of only KCl and were pooled from a total of nine independent experiments obtained using four different membrane preparations. DADLE and ICI 174864 were present in each experiment, whereas the number of observations for the other ligands are: ICI 154129, 5; naloxone (*NALOX*), 3; naltrexone (*NALTREX*), 4; MR 2266, 6; diprenorphine, 5; morphine, 3; and DPDPE, 2. *Points* are means whose standard errors (below 5% of the mean in all cases) are not shown for clarity. b, The decrease of GTPase in the absence (basal) or presence of each ligand as a function of log[Na^+/K^+] (shown in a) was fit by linear regression. The slope computed for each ligand (Na^+ effect on the *y*-axis) and its standard error are replotted as a function of the relative intrinsic activity of the ligand. Relative intrinsic activities were determined in only Na^+ for agonists (as ratio of the net stimulation produced by each agonist versus that produced by DADLE) and in only K^+ for the antagonists (as ratios of the net inhibition produced by each antagonist versus that observed in the presence of ICI 174864).

Mg^{2+}, accounted for a larger portion of inhibited activity, and was more pronounced in K^+ than in Na^+; this second component corresponds to the sodium effect on basal activity, which shows an identical Mg^{2+} dependency and is largely reduced in toxin-treated membranes (Fig. 7b).

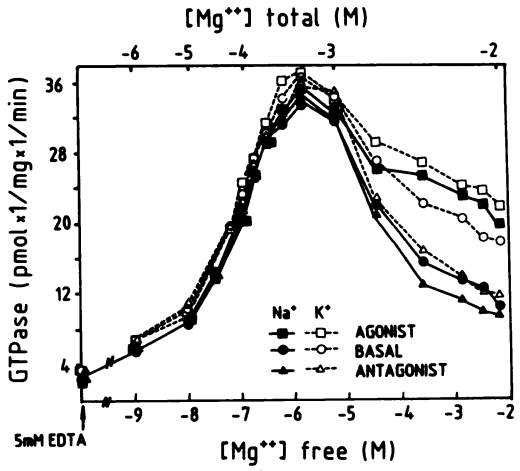

Fig. 6. Effect of Mg^{2+} on GTPase activity in membranes in the presence of either agonist or antagonist, studied in Na^+ or K^+. GTPase in NG108-15 membranes was assayed in Buffer B containing 1 mM EDTA and increasing concentrations of $MgSO_4$. The concentration of free Mg^{2+} was calculated as described in Experimental Procedures. The experiment was performed in the presence of 150 mM of either NaCl (*solid symbols*) or KCl (*open symbols*). Agonist (DADLE) or antagonist (ICI 174864) was present at 10 μM. The *points* on the *ordinate* were obtained in the presence of 5 mM EDTA and absence of added Mg^{2+}. These experiments are representative of two additional ones, one of which was performed using 0.2 mM EDTA; both gave comparable results.

To further characterize the two components of Mg^{2+}-dependent GTPase activity, we compared the basal rate of GTP hydrolysis as a function of $[Mg^{2+}]_{free}$ in intact or ADP-ribosylated G_i/G_o purified from bovine brain. Fig. 8 shows that the stimulatory effect of Mg^{2+} on the basal GTPase of G_i/G_o was slightly altered following pertussis toxin treatment. As free Mg^{2+} was increased between 10 nM and 1 μM, GTPase activity increased in a manner similar to that observed for membranes (Figs. 6 and 7) and, like in membranes, this increase was only 10–18% lower for toxin-treated proteins. In contrast to what observed in membranes, however, higher concentrations of Mg^{2+} did not further enhance the difference between toxin-treated and intact G proteins; instead, this difference was less evident at the highest concentration of metal. Replacement of Na^+ by K^+, at constant [Cl^-] (150 mM), had no effect on the GTPase activity of G_i/G_o over the entire range of free Mg^{2+} concentrations examined (Fig. 8). Thus, only a small reduction of the basal rate of GTP hydrolysis can be produced by ADP-ribosylation of purified G proteins and this reduction is comparable to that observed in membranes at submicromolar concentrations of Mg^{2+}. Neither the large toxin-induced diminution of basal activity observed at millimolar concentrations of Mg^{2+} nor the effect of Na^+ ions could be observed for purified G proteins.

The concentrations of pertussis toxin (offered to intact cells) necessary to induce in membranes reduction of the sodium effect on basal GTPase activity and suppression of receptor-dependent activity are compared in Fig. 9. There are no obvious differences in the potency of the toxin for these two effects. Pertussis toxin treatment did not abolish (Fig. 9, *inset*) the slight stimulation of low affinity nucleotidase produced by sodium (see Membranes and GTPase assay in Experimental Procedures), indicating that this cation effect is neither correlated with nor interferes with the sodium effect on high affinity GTPase.

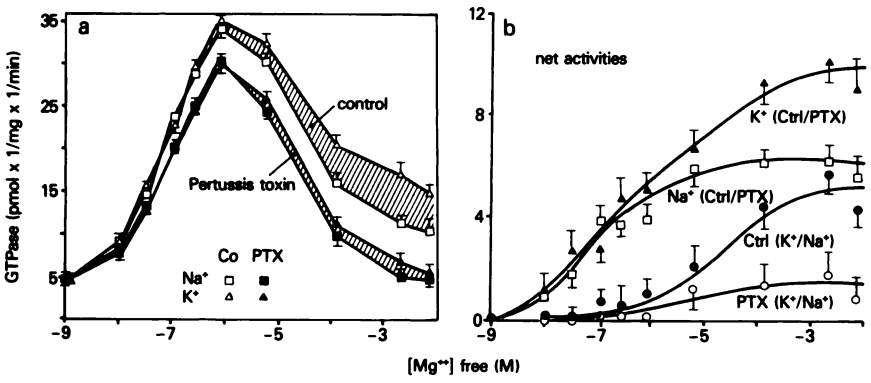

Fig. 7. Effect of Mg^{2+} on sodium regulation of basal GTPase in membranes treated or not with pertussis toxin. a, GTPase was assayed under conditions identical to those of Fig. 6, in membranes prepared from NG108-15 cells that had been exposed to either pertussis toxin (10 ng/ml, 24 hr) (*solid symbols*) or its diluent (*open symbols*). Assays contained 150 mM concentrations of either NaCl or KCl, as indicated. *Shaded areas* illustrate sodium effects in the two membranes. b, Data of a are replotted as net activities to illustrate how the GTPase activity suppressed by pertussis toxin treatment depends on the concentration of Mg^{2+}. Shown are net difference in activity between control and toxin-treated membranes measured in K^+ (▲) or in Na^+ (□) (total toxin effect) and net difference between activities in the presence of K^+ and Na^+ observed in control (●) or in toxin-treated (○) membranes. The standard errors of net activities are computed as described in Data analysis and expression of results in Experimental Procedures.

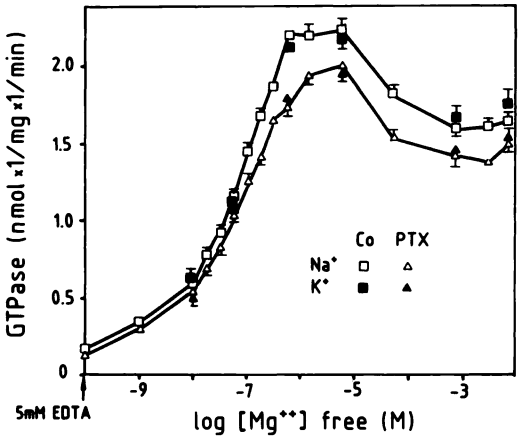

Fig. 8. Effect of Mg^{2+} on the basal rate of GTP hydrolysis by purified G_i/G_o with or without ADP-ribosylation by NAD^+ and pertussis toxin. G_i/G_o were preincubated in ADP-ribosylation reaction mixtures in the presence (*PTX*) or absence (*Co*) of pertussis toxin (20 μg/ml). The proteins were then assayed for GTPase in Buffer B, as described in Fig. 6, in the presence of 150 mM concentrations of either NaCl (*open symbols*) or KCl (*closed symbols*). G proteins were present in the final GTPase assay mixture at 45 ng/100 μl. The experiment was repeated using 0.2 mM EDTA with identical results.

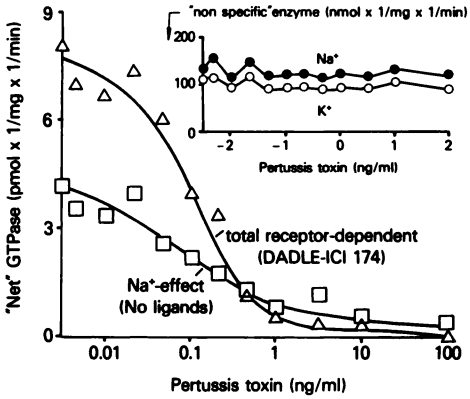

Fig. 9. Comparison of the effects of pertussis toxin on receptor-mediated and Na^+-mediated regulation of GTPase in membranes. Membranes were prepared from NG108-15 cells that had been exposed (18 hr) to increasing concentrations of pertussis toxin (*abscissa*). GTPase was assayed in Buffer B containing 10 mM $MgSO_4$ and 150 mM levels of either NaCl or KCl; DADLE and ICI 174864 were present at 10 μM. Net activities are shown. *Total receptor-dependent* is the difference in GTPase measured in the presence of agonist and in the presence of antagonist (in KCl). Net *Na^+ effect* is the difference in basal activity in the presence of K^+ and Na^+. *Inset*, low affinity GTP hydrolysis (measured in the presence of 50 μM GTP) determined in the same membranes in the presence of Na^+ or K^+. The apparent stimulation of this activity exerted by replacement of Na^+ by K^+ (see Experimental Procedures) is not abolished by pertussis toxin treatment.

Reconstitution of agonist and antagonist effects by purified G proteins in membranes inactivated by pertussis toxin. To study the reconstitution of the effects of agonists and antagonists by purified G proteins in membranes, cells were treated with pertussis toxin (10 ng/ml) to inactivate opioid-mediated regulation of GTPase and the resulting membranes were subsequently incubated with different concentrations of G proteins. In the absence of membranes, the GTPase activity of G_i/G_o increased linearly with increasing concentrations of protein and was not affected by the addition of either DADLE or ICI 174864 up to 10 μM (Fig. 10a, *inset*). When G_i/G_o were added to pertussis toxin-treated membranes, opioid-mediated stimulation was reconstituted in a specific fashion, inasmuch as no opioid effect was observed using G proteins previously exposed to heat or if the G proteins had been ADP-ribosylated by pertussis toxin (Fig. 10a). To examine whether addition of G protein could restore the effects of both opioid ligands and sodium on basal activity, we carried out reconstitution experiments in the presence of either NaCl or KCl. The effect of replacement of Na^+ by K^+ on the "basal" activity of the membrane cannot be directly measured under these conditions, because it is obscured by the large increase in overall GTPase activity that results from the addition of purified G proteins (Fig. 10a). However, the effect of sodium on basal activity can be extrapolated indirectly from the relative proportions of the effect of agonists and antagonists in the presence of Na^+ and K^+, respectively. Fig. 10b shows that, when reconstitution was carried out in the presence of Na^+, very little effect of the antagonist was present, inasmuch as the total receptor-dependent activity (i.e., activity in the presence of agonist minus that in the presence of antagonist) was very similar to the net activity stimulated by the agonist. In contrast, in the absence of sodium (only K^+ present), the effect of the antagonist was clearly detectable (Fig. 10c). This indicates that the addition of purified G proteins to membranes whose receptor had been previously uncoupled by treatment with pertussis toxin can restore not only agonist-mediated stimulation but

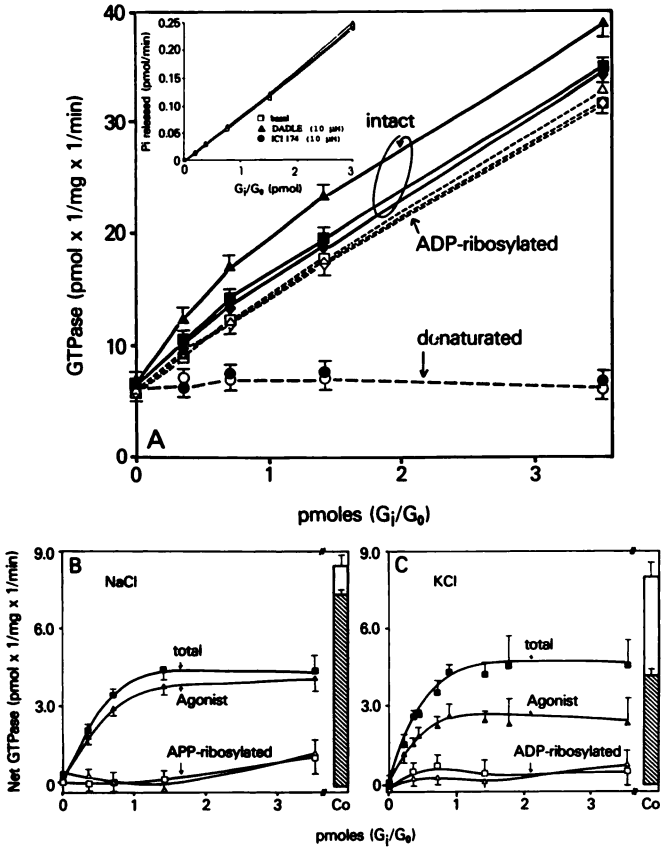

Fig. 10. Reconstitution by G_i/G_o of receptor-mediated regulation of GTPase in membranes obtained from NG108-15 cells treated with pertussis toxin. NG108-15 cells were pretreated with pertussis toxin (10 ng/ml, 24 hr) or its diluent and membranes were prepared. a, Membranes (10 μg) were mixed with different amounts of G_i/G_o (as indicated). G proteins had been exposed to an ADP-ribosylation mixture in the absence (*intact*, ▲, ■, ♦) or presence (*ADP-ribosylated*, △, □, ◇) of pertussis toxin or preincubated at 95° for 5 min (*denatured*, ● ○). GTPase was assayed in a total volume of 100 μl (see Experimental Procedures for details) in NaCl (150 mM) in the presence of 10 μM levels of either DADLE (▲, △, ●) or ICI 174864 (♦, ◇, ○) or in their absence (■, □). *Inset*, the GTPase activity of G_i/G_o in the absence of membranes is not affected by DADLE or ICI 174864. b and c. Experiments similar to those of a performed in 150 mM concentrations of either NaCl (b) or KCl (c). Data are shown as net activities. *Total*, GTPase in the presence of DADLE minus that in the presence of ICI 174864 (control, ■; ADP-ribosylated, □); net agonist-mediated (*Agonist*); GTPase in the presence of DADLE minus that in the absence of added ligand (▲, △). Their difference is the net antagonist-mediated effect. The *bars* indicate the corresponding total (□) and net agonist effect (▨) measured in parallel in membranes prepared from cells not exposed to pertussis toxin.

also the mechanism responsible for the spontaneous activation of basal activity and its regulation by sodium ions.

The extent of activity that could be "reconstituted" in these experiments is only 50–60% of that observed in control membranes (i.e., in membranes prepared from cells incubated in the absence of toxin and assayed in parallel). In the presence of either Na^+ or K^+, this reconstituted activity was saturable with respect to the concentration of G protein and reached a plateau at about 1–2 pmol of G_i/G_o added (10–20 nM). Because 10 μg of NG108-15 membranes contain 200–300 fmol of G_i/G_o,[2] it appears that a 5–10-fold excess of purified G protein is necessary for the partial restoration of receptor-dependent activity under this assay condition.

Discussion

In this study we show that opioid antagonists can inhibit basal GTPase activity in NG108-15 membranes, when this activity has been enhanced by replacement of sodium ions by potassium at constant ionic strength.

The study of GTPase activity in intact membranes is made difficult by several factors, one of which is lack of knowledge of what "basal" low K_m activity measured in membrane actually represents. Cassel and Selinger (21, 37), in their work on catecholamine-stimulated GTPase in turkey erythrocytes, suggested that basal and hormone-stimulated activities might be two different molecular entities, because only the latter was suppressed by *N*-ethylmaleimide or cholera toxin treatment of membranes. Recent studies on purified G_i/G_o indicate that the intrinsic GTPase and guanine nucleotide-binding activities of these proteins are not dramatically altered by treatment with either pertussis toxin (11, 38) or *N*-ethylmaleimide (39) and suggest that the marked inhibition exerted by these agents on receptor-stimulated activities observed either in membranes or in reconstituted systems is due to a selective suppression of the ability of G proteins to be activated by receptors (38).

In a series of studies on opioid-stimulated GTPase in NG108-15 cell membranes, we accumulated evidence that a substantial portion of basal activity in this system represents preactivated G proteins (19). Part of this apparent preactivation of basal activity may result from an opioid receptor-dependent mechanism, inasmuch as small but significant reductions of basal activity were also observed following either opioid agonist-mediated down-regulation (40) or alkylation of receptors with an irreversible antagonist (19).

The fact that a number of reversible opioid antagonists, as documented here and elsewhere (22), can stereospecifically inhibit basal GTPase in NG108-15 membranes is a further indication that a substantial portion of this activity may indeed reflect a precoupled G protein-opioid receptor complex. The effect (inhibitory) of the antagonist on basal GTPase was symmetrical and reciprocal to the stimulatory effect of the agonist, and both occurred with no detectable time lag. The fact that ICI 174864 does not alter the GTPase of purified G proteins indicates that its inhibitory effect in membranes is receptor mediated and does not result from a nonspecific direct interaction with G proteins. We have also shown that the effect of the antagonist on basal GTPase rate cannot be attributed to contamination of membranes by endogenous opioid agonists or to the hypothetical formation of a long-lived agonist-bound form of the receptor that may occur during cell growth or preparation of membranes (22). We further show here that opioid antagonists exhibit a wide spectrum of apparent negative intrinsic activities for GTPase when compared under conditions of maximal receptor occupancy (i.e., under conditions in which their different affinities for the receptor play no role). There is no obvious relation between apparent receptor affinity of these ligands and their maximal inhibitory effect on GTPase.[3] Instead, a close correlation would have been expected

[2] The content of G_i/G_o in NG108-15 membranes was measured by quantitative immunoblot with a pan-reactive antiserum as described elsewhere (J. Lang and T. Costa, submitted for publication).

[3] Although ICI 154129 exhibited 5–10-fold lower affinity than ICI 174864, naloxone, naltrexone, and MR 2266 had affinities 2–4-fold higher than ICI 174864. Another benzomorphan antagonist, Win 444441, which like MR 2266 was devoid of negative intrinsic activity for GTPase, had a 10-fold higher affinity than ICI 174864. Apparent affinities were measured by competition for the binding sites labeled by the partial agonist [^{3}H]diprenorphine in the presence of NaCl (unpublished data).

if inhibition reflected their competition with an endogenous agonist that stimulated GTPase.

Additional information on the nature of the mechanism underlying this apparent stimulated state of basal GTPase in membranes and its inhibition by opioid antagonists comes from studies of its dependence on ionic milieu. Klee and co-workers (23) pointed attention to the essential role of ions like Na^+ and Mg^{2+} on agonist-mediated GTPase stimulation in intact membranes. These effects parallelled similar requirements for opioid-mediated inhibition of adenylate cyclase and for establishment of high affinity opioid agonist binding (41, 42). We show here that monovalent ions can produce two distinct specific effects on GTPase in membranes, when inhibition by ionic strength is properly subtracted. The first is stimulation, which is evident when the net difference in activity between agonist and antagonist (total receptor-dependent activity) is examined. This occurs at low salt concentrations and exhibits an apparent specificity for "alkaline" cations because K^+ and Na^+ produce similar maximal stimulations, whereas *N*-methylglucamine is much less effective. The mechanism for this effect is not clear, but it may be similar to that previously characterized in reconstituted systems, where millimolar concentrations of either KCl or NaCl not only increased the total portion of G protein that could be activated by receptor but also enhanced the rate constant for receptor-G protein interaction in liposomes (43). The second type of cation effect observed here exhibits a clear Na^+/K^+ specificity, inasmuch as Na^+ (and to a lesser extent Li^+) but not K^+ can alter the relative proportions of agonist-stimulatable and antagonist-inhibitable GTPase activities. This sodium-selective effect is best observed when the total concentration of cations and anions is held constant and the only variable altered is the ratio Na^+/K^+. Under these conditions, sodium selectively reduces basal GTPase activity by increasing the net effect of the agonist, reciprocally decreasing that of the antagonist, and leaving total receptor-dependent activity unaltered. This suggests that sodium does not change the total proportion of G proteins that can be activated or inactivated when receptors are occupied by agonists or antagonists, respectively, but instead reduces the fraction of G proteins that are under tonic stimulation in the absence of ligand.

Further understanding about the nature of basal GTPase activity in membranes comes from investigations of its dependence on Mg^{2+}. As we show here, there are two clearly distinguishable effects of the divalent metal.

The first is stimulation (10–20-fold), which occurs at nanomolar concentrations of free Mg^{2+}, and it is strikingly similar in membranes and purified G_i/G_o, when the two preparations are assayed under comparable conditions. Pertussis toxin treatments of either membranes or purified proteins produce only small and similar decreases (10–20%) of this Mg^{2+} stimulation; for this range of Mg^{2+} concentrations there are no effects of agonists or antagonists on GTPase in membranes, nor are there effects of Na^+. This stimulation is conceivably mediated by a direct interaction between Mg^{2+} and G protein and reflects the well known fact that hydrolysis of GTP by all types of G proteins required nanomolar concentrations of Mg^{2+} (1, 44, 45). Thus, the activity measured under this low range (0.01–1 μM) of Mg^{2+} concentrations more closely represents the true basal activity of the G proteins present in the membranes. Because replacement of Na^+ by K^+ does not affect this activity in membranes or in soluble purified G proteins, it is clear that sodium does not alter the true basal GTPase activity of G proteins.

The second effect of Mg^{2+} is inhibition. It occurs at concentrations above 10 μM and reaching the millimolar range. At these concentrations, Mg^{2+} "promotes" both receptor- and sodium-mediated regulations of GTPase in intact membranes but not in those prepared from cells previously exposed to pertussis toxin. Furthermore, in this same range of Mg^{2+} concentrations, the difference in apparent basal activity between control and ADP-ribosylated G proteins in membranes (but not in purified preparations) becomes increasingly larger. This indicates that receptor-G protein interaction, sodium-specific effects, and apparent activation of basal activity have an identical type of Mg^{2+} dependence and suggests that these three processes are manifestations of a common mechanism. Moreover, the *in vivo* concentrations of pertussis toxin necessary to abolish receptor-mediated control of GTPase and Na^+-mediated regulation of basal activity are identical (IC_{50}, 0.1 ng/ml in both cases).

Thus, the direct and most simple interpretation of the data presented here is that receptor and G protein can establish a spontaneous interaction in the membrane (when free Mg^{2+} is >10 μM) and this results in a tonic level of GTPase stimulation (apparent basal activity). This interaction can be reduced by antagonists which negative efficacy and modulated specifically by sodium ions. The large decrease of basal GTPase activity observed at millimolar Mg^{2+} concentrations in membranes prepared from cells pretreated with pertussis toxin is primarily attributable to "uncoupling" of this spontaneous interaction of receptor and G protein and, secondarily, to the slightly reduced basal rate of hydrolysis that ADP-ribosylated G proteins exhibit both in membranes and in solution. (This slight reduction of basal rate explains why toxin-treated membranes display a GTPase activity slightly lower than control membranes, even in the presence of maximal concentrations of antagonist and sodium or in the presence of submicromolar Mg^{2+}, i.e., under conditions in which GTPase due to receptor-G protein precoupling is inhibited.)

A further indication for the link between antagonist-mediated inhibition and Na^+ regulation of basal GTPase is given by reconstitution experiments. As shown in the present study, in membranes in which both agonist- and antagonist-mediated effects on GTPase had been abolished by pertussis toxin treatment, the addition of purified G proteins restores both processes, provided that K^+ ions replace Na^+ ions in the reaction. Thus, receptor-mediated control of GTPase and sodium regulation of basal activity can be reconstituted in parallel.

We show in this study that there is striking relationship between the relative intrinsic activity (defined as maximal effect on GTPase) of a given opioid ligand and the sodium sensitivity of the GTPase activity elicited upon receptor occupation by that ligand. The more the intrinsic activity differs from zero, the less the GTPase activity is sensitive to sodium (Fig. 5), and this is true regardless of the direction, positive or negative, of the intrinsic activity of the ligand. Assuming that the intrinsic activity of each ligand reflects its ability to induce binding of the receptor to the G protein, then the proportion of receptor-dependent GTPase activity in membranes is maximal in the presence of a full agonist and minimal in the presence of a negative antagonist. If, as previously suggested (23, 46), the effect of sodium resulted from the ability of the cation to inhibit basal activity more strongly than receptor-

stimulated activity, then GTPase activity should be maximally Na^+ sensitive in the presence of the negative antagonist and minimally in the presence of the agonist, and the relation between magnitude of Na^+ effect and intrinsic activity should be monotonic. Instead, we found this relation to be bell-shaped, because the Na^+ sensitivity of GTPase in the presence of full agonist or negative antagonist was much lower than in the presence of partial agonists or antagonists or in the absence of ligand. These findings may suggest that the mechanism of receptor-mediated activation of G proteins in intact membranes involves preformed receptor-G protein complexes that are activated by agonists and inactivated by antagonists, rather than "freely floating" dissociated components whose ability to interact with each other is enhanced by agonists and decreases by negative antagonists.

Although these data strongly suggest that basal GTPase activity in membranes is actually a "stimulated" activity, they do not allow one to distinguish between two possible mechanisms. The first is that this apparent activation is due to a complex between "empty" opioid receptors and G protein, possibly modulated by a site for Na^+ on the receptor. A second is that constitutive stimulation of G proteins in the membrane results from an additional sodium-sensitive component, which is inhibited by antagonist-occupied opioid receptors. Two pieces of evidence presented here speak against this second possibility.

(a) ICI 174854 did not block the stimulatory effect of bradykinin on GTPase, suggesting that the inhibition mediated by opioid antagonists described in this study is limited to a subpool of G proteins that is under opioid receptor control. However, there is controversy as to whether bradykinin-stimulated GTPase is pertussis toxin sensitive (33, 34). Although NG108-15 cells possess in their membranes additional receptors that can interact with either G_i/G_o (muscarinic and α_2-adrenergic) or G_s (prostaglandin E_1 and secretine), we did not observe a reproducible stimulation of GTPase with any of those ligands, and we could not further test whether opioid antagonists can suppress the GTPase activity stimulated by receptors other than opioid.

(b) The effect of ICI 174864 and that of pertussis toxin treatment on basal activity in membranes prepared from cells cultured in the total absence of serum and growth factors were of comparable magnitude to those observed in serum-exposed cells. These findings indicate that basal GTPase is not preactivated by a growth factor receptor-related mechanism in NG108-15 cells (47). However, they do not rule out the possibility that an as yet unknown endogenous membrane component activates G proteins in these cells.

Our data do not allow for a distinction between the two mechanisms discussed above, but they demonstrate the close relationship between apparent activation of basal GTPase activity and effect of Na^+ ions. Thus, the elucidation of the mechanism underlying activation of basal GTPase activity in NG108-15 cells will require the identification of the site of action of Na^+ ions. As shown here, Na^+ has no direct effect on purified G proteins, suggesting that the binding site of this cation is not on one of their subunits. Similar conclusions have been reached previously (17, 29, 47, 48). If the apparent stimulation of basal GTPase activity results from the spontaneous interaction between unoccupied opioid receptors and G proteins, then the site of action of sodium would be on the receptor itself. In this case the relationship between relative intrinsic activity and sodium sensitivity of the GTPase elicited by each ligand (Fig. 5) would represent the relative ability of antagonists or agonists to stabilize the receptor into a range of conformations that extends from "fully inactive" to "fully active." This wide range of intrinsic activities has been documented previously for ligand-mediated gating of ion channels like γ-aminobutyrate receptor-operated chloride channels (49) or dihydropyridine-sensitive Ca^{2+} channels (50), but not yet for G protein-linked receptors. If, instead, a still unknown sodium-sensitive molecular component interacts with G proteins and receptor in intact membranes (18), this component should exert a major constraint on the ability of receptors to interact with G proteins. The continuous spectrum from negative to positive values of the intrinsic activities of opioid ligands observed here would then reflect the relative abilities of different ligands to free receptors from this molecular constraint and promote their interaction with G proteins. Studies with purified receptors and G proteins in synthetic membranes will be essential to clarify whether only these two components are sufficient to establish a sodium-sensitive antagonist-inhibitable basal GTPase activity. Our data indicate that sodium-specific regulation of receptor and G protein interactions is a criterion of reconstitution of receptor function observed in native membranes that should no longer be neglected in those type of studies.

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