Cross Talk between Stimulatory and Inhibitory Guanosine 5′-Triphosphate Binding Proteins: Role in Activation and Desensitization of the Adenylate Cyclase Response to Vasopressin†

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ABSTRACT: The inhibitory GTP-binding protein (G_i) is known to mediate the effects of a number of hormones that act through specific receptors to inhibit adenylate cyclase. In this study we examined the mechanism whereby G_i modulates the response of adenylate cyclase to a stimulatory hormone and its role in desensitization. In membranes prepared from the cultured renal epithelial cell line $LLCPK_1$, adenylate cyclase activity was stimulated 16-fold by 1–2 μM lysine vasopressin. Addition of GTP (1–100 μM) resulted in stimulation of basal activity but inhibition of hormone-stimulated activity ($\sim$40% inhibition at 100 μM GTP). This constrasts with the usual effect of GTP to support or augment activation by stimulatory receptors. The inhibitory effect was abolished by pertussis toxin, which had little effect on basal activity in the absence or presence of added GTP or on vasopressin-stimulated activity in the absence of added GTP. GTP-mediated inhibition was vasopressin concentration dependent. At concentrations of vasopressin below the $K_{1/2}$ for enzyme activation (approximately 0.6 nM), GTP was stimulatory, and at higher concentrations, GTP was inhibitory. The inhibitory effect of GTP was also observed for a V_2-receptor agonist and was not abolished by a V_1-receptor antagonist, indicating that a distinct V_1 receptor did not mediate inhibition of adenylate cyclase. Using the known subunit structure of adenylate cyclase, we developed the minimal mechanism that would incorporate a modulatory role for G_i in determining net activation of adenylate cyclase by a stimulatory hormone. The predicted enzyme activities for basal and maximal hormone stimulation in the presence and absence of GTP were generated, and model parameters were chosen to match the experimental observations. Direct interaction of the receptor with G_i was not required, but a direct inhibitory interaction of the α_i subunit with G_i was necessary for the model to yield the experimentally observed effects of GTP on basal and hormone-stimulated enzyme activity. Elimination of G_i input in the model without changing any other model parameters mimicked the experimentally observed effects of pertussis toxin. Despite the lack of a requirement for direct interaction of the receptor with G_i, the model predicted the inhibitory effect of GTP to be hormone concentration dependent. We also observed that forskolin-stimulated activity was inhibited by GTP, an effect that could be reversed by pertussis toxin, further supporting that interaction of the vasopressin receptor with G_i was not required for GTP-mediated inhibition. The effects of pertussis toxin and GTP in desensitized cells observed experimentally were compared with the pattern predicted by the model. Pertussis toxin pretreatment did not affect homologous desensitization, excluding a role for G_i in homologous desensitization. The results obtained for desensitized cells were predicted by postulating a defect in hormone-stimulated guanyl nucleotide exchange on G_s in the model. These results allow us to propose a comprehensive mechanism whereby cross talk between G_i and G_s determines net adenylate cyclase activation by a stimulatory hormone. The modulatory role of G_i does not require its direct interaction with the receptor but does require a direct inhibitory interaction with the catalytic unit. The results also exclude a direct role for G_i in homologous desensitization.

The action of a number of hormones that inhibit adenylate cyclase is now known to be mediated by receptor coupling to a distinct GTP[1]-binding protein, G_i (Gilman, 1984). Recently, studies using detergent-solubilized and phospholipid-reconstituted systems have suggested an additional role for G_i in modulating overall adenylate cyclase activation by stimulatory hormones (Cerione et al., 1985a,b). Whether this modulatory role of G_i involves a direct interaction with the stimulatory receptor or with the catalytic subunit has not been determined. Furthermore, an overall kinetic mechanism for the modulatory action of G_i in the net activation of adenylate cyclase by a stimulatory hormone, based on studies in an intact membrane, has not been developed. Recent attempts to de-

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[1] Abbreviations: G_i, inhibitory GTP-binding protein; G_s, stimulatory GTP-binding protein; α_s, α-subunit of G_s; α_i, α-subunit of G_i; β, β-subunit of G_s and G_i; C, catalytic unit of adenylate cyclase; H, hormone; R, receptor; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; ADP, adenosine 5′-diphosphate; cAMP, adenosine cyclic 3′,5′-monophosphate; LVP, lysine vasopressin; dDAVP, deamino[8-D-arginine]vasopressin.

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velop an adenylate cyclase activation model based on intact membrane studies have not incorporated the subunit structure of the GTP-binding proteins or the possible modulatory influence of G_i, which have only since been appreciated (Tolkovsky et al., 1982; MacFarlane, 1982; Blum, 1984).

In the pig kidney epithelial cell line $LLCPK_1$, as in a number of other urinary epithelia, vasopressin stimulates adenylate cyclase through interaction with its V_2 receptor (Goldring et al., 1978; Roy et al., 1981). In this study we show that the addition of GTP in the concentration range of 1–100 μM inhibits vasopressin-stimulated adenylate cyclase activity. This inhibition is dependent upon the concentration of vasopressin, is not mediated by a separate V_1 receptor, and is entirely reversed by pertussis toxin, which inactivates G_i (Katada & Ui, 1982a,b). Furthermore, GTP-mediated inhibition was not involved in desensitization of the hormone response.

On the basis of these results, we developed the minimal model that would incorporate a modulatory role for G_i in determining overall activation of adenyate cyclase by a stimulatory hormone and chose model parameters that yielded the observed hormone concentration dependent effects of GTP. The modulatory influence of G_i did not require direct interaction with the receptor but did require a direct inhibitory influence of the α-subunit of G_i on catalytic activity. This model provides a mechanistic basis for the varied effects of GTP to either support or inhibit activation of adenylate cyclase by stimulatory receptors as has been described in different systems (Rodbell et al., 1971; Boeckert et al., 1972; Cooper et al., 1979; Owens et al., 1985). In the appendix we provide the explicit mathematical formulation for the model.

Experimental Procedures

Cell Culture. $LLCPK_1$ cells, an established epithelial cell line derived from pig kidney (Hull et al., 1976), were maintained in 10-cm polystyrene tissue culture dishes with Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cells were maintained in an incubator at 37 °C and aerated with 95% air–5% CO_2. Experiments were conducted on confluent cultures, and media were changed 24 h prior to experiments. Where indicated, pertussis toxin was added at the time of this medium change at a concentration of 100 ng/mL.

Adenylate Cyclase Assay. Cells were washed with a phosphate buffered balanced salt solution, pH 7.4, and scraped into cold 5 mM Tris-HCl, pH 7.4, and 1 mM EGTA and disrupted with a Dounce homogenizer (15 strokes). The homogenate was spun at 50*g* for 10 min to remove whole cells and nuclei and then respun at 10000*g*. The 10000*g* pellet was resuspended in cold 5 mM Tris-HCl, pH 7.4, and 0.5 mM EGTA and kept on ice until initiation of the assay. Adenylate cyclase activity was assayed by measuring the rate of conversion of [α-^{32}P]ATP to [α-^{32}P]cAMP as previously described (Roy et al., 1981). The incubation medium contained 50 mM Tris-HCl, pH 7.4, 0.2 mM EGTA, 0.1 mM cAMP, 0.1 mM ATP, 5 mM $MgCl_2$, 0.5 mg/mL creatine kinase, 1.5 mg/mL creatine phosphate, 3 μg/mL adenosine deaminase, 10–20 μCi/mL [α-^{32}P]ATP, and other activators (hormone, GTP) indicated under Results in a final volume of 50 μL/assay tube containing 10–20 μg of homogenate protein. Incubation time was 20 min at 30 °C during which time cAMP generation was linear. The reaction was stopped and [^{32}P]cAMP measured as previously described (Roy et al., 1981). Each measurement was performed in triplicate and adenylate cyclase activity expressed as pmol of cAMP (mg of protein)$^{-1}$ (5 min)$^{-1}$. Where indicated under Results (Desensitization Studies), cells were preincubated in phosphate-buffered saline, pH 7.4, in the absence or presence of 200 nM LVP at 37 °C for 20 min prior to assay.

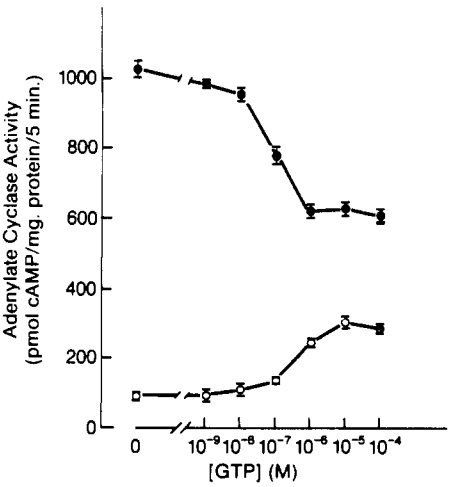

FIGURE 1: Effect of GTP addition on adenylate cyclase activity in $LLCPK_1$ cells. Basal (open circles) and 2 μM vasopressin-stimulated (closed circles) adenylate cyclase activity was measured with the addition of each of the concentrations of GTP indicated. Results are mean ± SEM of triplicate deteminations in this experiment, which is representative of four.

Model Formulation. See Appendix.

Materials. Radiochemicals were obtained from New England Nuclear (Bedford, MA). Cell culture media, sera, and enzymes were obtained from GIBCO (Grand Island, NY). Pertussis toxin was obtained from List Biochemicals (Campbell, CA). GTP, forskolin, and adenosine deaminase were obtained from Boehringer-Mannheim (Indianapolis, IN). dDAVP was obtained from Fering Pharmiceuticals (Malmo, Sweden). 1-(β-Mercapto-β,β-cyclopentamethylenepropionyl)-2-(*O*-methyltyrosyl)[Arg^8]vasopressin was obtained from Penninsula Laboratories (Belmont, CA). Lysine vasopressin and other compounds were obtained from Sigma Chemical Co. (St. Louis, MO) and were of reagent grade.

Results

The addition of GTP to the adenylate cyclase incubation medium resulted in concentration-dependent stimulation of basal activity but inhibition of maximal hormone-stimulated activity (Figure 1). Approximately 40% inhibition of maximal hormone-stimulated activity occurred with addition of 1–100 μM GTP. The hormone concentration dependence for the inhibitory effect of GTP is shown in Figure 2. At concentrations of hormone below the $K_{1/2}$ for enzyme activation, GTP addition enhanced enzyme activity while at higher hormone concentrations GTP was inhibitory. The inhibitory effect of GTP was reversed by pretreatment of the cells with pertussis toxin (Figure 3), suggesting that inhibition was mediated through G_i.

As indicated in Table I, adenylate cyclase activity stimulated by the V_2 receptor specific analogue dDAVP was inhibited by GTP, and this inhibition was reversed by pertussis toxin, indicating that the V_1 receptor was not involved. Furthermore, up to a 50-fold excess of the selective V_1-receptor antagonist 1-(β-mercapto-β,β-cyclopentamethylenepropionyl)-2-(*O*-methyltyrosyl)[Arg^8]vasopressin had no effect on adenylate cyclase activity and correspondingly did not abolish GTP-mediated inhibition (data not shown). These results excluded the possibility that a distinct V_1-receptor subtype mediated the inhibitory effects of GTP, in a manner analogous to other adenylate cyclase inhibiting receptors (e.g., α_2-adrenergic). Adenosine could not have been responsible for GTP-mediated

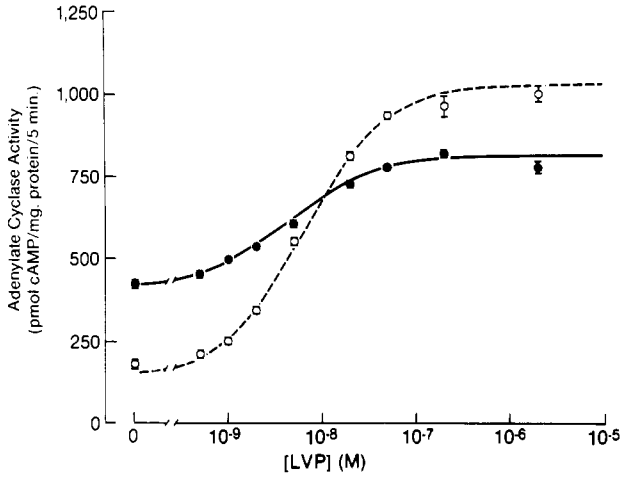

FIGURE 2: Experimentally observed hormone concentration dependence of GTP-mediated inhibition. Adenylate cyclase activity was determined at each concentration of vasopressin shown without (open circles, broken line) or with (closed circles, solid line) the addition of 100 μM GTP. Each determination represents the mean ± SEM of triplicate determinations in this experiment, which is representative of four. When fitted to a single site binding model, the $K_{1/2}$ for activation in the absence of GTP (broken line) was 0.65 ● 0.07 nM and in the presence of GTP (solid line) was 0.47 ± 0.04 nM.

Table I: GTP-Mediated Inhibition of dDAVP-Stimulated Adenylate Cyclase Activity[a]

GTP	pertussis toxin	adenylate cyclase activity [pmol of cAMP (mg of protein)$^{-1}$ (5 min)$^{-1}$]
−	−	1414 ± 169
+	−	761 ± 91[b]
+	+	2161 ± 302

[a] Results shown are the mean ± SEM of seven separate experiments. A concentration of 2 μM dDAVP was used in each assay. Where indicated, 100 μM GTP was added to the assay, and cells were pretreated with 100 ng/mL pertussis toxin in the culture medium the day prior to the experiment. [b] Significantly less than both other values ($p < 0.01$).

inhibition either, since adenosine deaminase was included in the incubation medium and methylxanthines, which serve as competitive antagonists at the adenosine receptor, were without effect. These results suggested that the observed modulation of vasopressin-stimulated adenylate cyclase activity by G_i did not involve a distinct inhibitory receptor subtype.

These results led us to develop a model that would incorporate G_i cross talk with G_s in the overall adenylate cyclase activation mechanism. The model is described under Discussion and its mathematical formulation given in the Appendix. Model parameters were chosen on the basis of the cumulative results for the effects of GTP on basal and maximal hormone-stimulated activity in all the experiments (Table II, left panel). Addition of 100 μM GTP resulted in approximately 2–3-fold stimulation of basal activity and 40–50% inhibition of maximal hormone stimulation. This same pattern (Table III, left panel) was obtained in the model using parameters that required no direct interaction of G_i with the receptor but that did require a direct inhibitory interaction with the catalytic unit. The cumulative results for the effects of pertussis toxin pretreatment are summarized in the right panel of Table II. Pertussis toxin pretreatment had little effect on basal or GTP-stimulated activity in the absence of hormone or on hormone-stimulated activity in the absence of GTP. However, in contrast to the inhibitory effect of GTP on hormone-stimulated activity, there was significant further stimulation by GTP in pertussis toxin pretreated cells. This same pattern of results (Table III, left panel) was predicted by the model, using the same parameters that were used to generate the left-hand side of Table III but with G_i input eliminated.

Since the results in Table III did not require direct interaction of G_i with the receptor, it was of interest to determine whether the hormone concentration dependent effects of GTP shown in Figure 2 could also be explained with the same model parameters, without requiring direct receptor–G_i interaction. By use of these parameters, the theoretically predicted vaso-

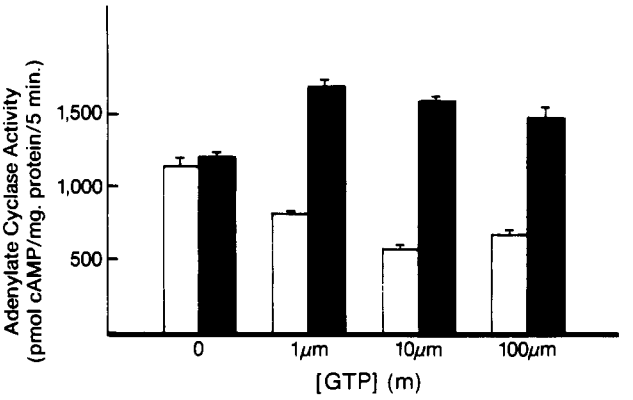

FIGURE 3: Reversal of GTP-mediated inhibition by pertussis toxin. Cells were preincubated in the absence (open bars) or presence (closed bars) of pertussis toxin (100 ng/mL) for 12 h prior to assay. Adenylate cyclase activity was assayed in the presence of 2 μM lysine vasopressin at each concentration of GTP indicated. Each result represents the mean ± SEM of triplicate determinations in this experiment, which is representative of three.

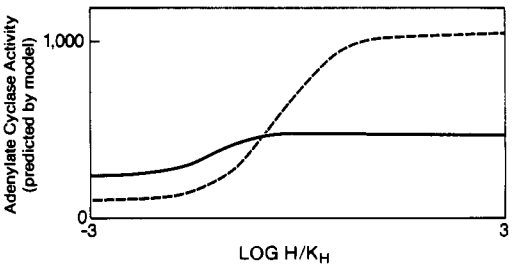

FIGURE 4: Model prediction for hormone concentration dependence of GTP-mediated inhibition. Parameters used were the same as those listed in Table III. Basal activity in the absence of GTP was set at 100 and maximal hormone stimulation at 10-fold. The predicted relative activity at each concentration of hormone in the absence (broken line) and presence (solid line) of GTP was determined by solving the model equations described in the Appendix for varying H/K_H.

Table II: Effects of GTP and Pertussis Toxin on Basal and LVP-Stimulated Adenylate Cyclase Activity[a]

	without pertussis toxin		with pertussis toxin	
	no GTP	100 μM GTP	no GTP	100 μM GTP
basal	84 ± 10 (15)	350 ± 46 (12)	74 ± 7 (12)	500 ± 55 (9)
2 μM LVP	1362 ± 122 (16)	837 ± 73 (16)[b]	1437 ± 137 (13)	1782 ± 113 (13)[b]

[a] The table summarizes all the results obtained for each incubation condition shown. Each results represents the mean ± SEM for the number of individual experiments shown in parentheses. For each experiment, adenylate cyclase activity was determined in triplicate and is expressed as pmol of cAMP (mg of protein)$^{-1}$ (5 min)$^{-1}$. [b] Significantly less than corresponding "no GTP" ($p < 0.01$).

Table III: Model Predictions for Effect of GTP on Adenylate Cyclase Activity[a]

	with G_i input		G_i input eliminated	
	–GTP	+GTP	–GTP	+GTP
basal	100	245	100	304
VP	1065	483	1081	1727

[a] Basal activity was set at 100 and model parameters chosen to give a 10-fold stimulation with maximal hormone (VP), approximately 2–3-fold stimulation of basal activity with GTP, and ~50% inhibition of hormone-stimulated activity with GTP. Actual values of the parameters used were as follows: $[\alpha_S]_T = 1$, $[\alpha_i]_T = 10$, $[\beta]_T = 10$, $[C]_T = 1$, $[R]_T = 1$, $K_b = 1$, $K'_b = 0.1$, $K_c = 0.001$, $K'_c = 0.0001$, $K_H = K_{HR} = K'_{HR} = 1$, $K_{hr} = K'_{hr} = \infty$, $k_1 = 100$, $k_2 = 1$, $k_3 = 20$, $k'_G = 2$, $k_g = 10$, and $k'_g = 0$. GTP addition was incorporated in the model as a 5-fold enhancement of the rate of GTP-dependent reaction steps. G_i input was eliminated (right side of Table) by setting $K'_c = 0.01$ (see Discussion and Appendix).

Table IV: G_i-Mediated Inhibition of Forskolin-Stimulated Adenylate Cyclase Activity[a]

	no pertussis toxin	pertussis toxin
no GTP	1996 ± 105	2193 ● 139
100 μM GTP	1128 ± 45[b]	2335 ± 266

[a] Adenylate cyclase activity was determined in triplicate in the presence of 100 μM forskolin in the absence or presence of added 100 μM GTP. Where indicated, cells were pretreated with pertussis toxin 12 h prior to assay. Results are expressed as pmol of cAMP generated (mg of protein)$^{-1}$ (5 min)$^{-1}$ and represent the mean ± SEM for five separate experiments. [b] $p < 0.01$ for comparison with all other incubation conditions.

pressin concentration dependence in the absence and presence of GTP was generated as described in the Appendix. The results shown in Figure 4 demonstrate that GTP-mediated inhibition is expected to be hormone concentration dependent as was experimentally observed with resultant flattening of the curve shape as well as leftward shift in the $K_{1/2}$ for enzyme activation with the addition of GTP. This indicates that a direct interaction of G_i with the stimulatory receptor is not needed to account for the hormone concentration dependency of GTP-mediated inhibition. This is further supported by the ability of GTP to inhibit forskolin-stimulated activity (bypassing the receptor) as shown in Table IV and is in agreement with previous reports in other systems (Aktories et al., 1983; Seamon & Daly, 1982).

Prior exposure of intact cells to vasopressin results in the attenuation of subsequent adenylate cyclase responsiveness to vasopressin (Roy et al., 1981; Figure 5, top panel: 7 vs. 3). Since the magnitude of attenuation of adenylate cyclase responsiveness in the desensitized state is similar to that achieved by GTP addition to nondesensitized membranes, it was of interest to evalute the possible role of G_i in desensitization with pertussis toxin pretreatment and GTP addition. As evident in the top panel of Figure 5, pertussis toxin pretreatment failed to abolish desensitization (15 vs. 11) but was clearly effective in overcoming the inhibitory effect of GTP addition in both nondesensitized (12 vs. 4) and desensitized cells (16 vs. 8). This result does not support a role for G_i in mediating homologous desensitization in this sytem and is further consistent with the absence of a direct interaction of the receptor with G_i.

In other systems, homologous desensitization has been attributed to a diminished ability of hormone-occupied receptor to accelerate guanyl nucleotide exchange (GTP–GDP interconversion) on G_s (Solomon et al., 1984). By use of the same model parameters used to generate Table III, the predicted effects of a 50% decrease in the rate of guanyl nucleotide

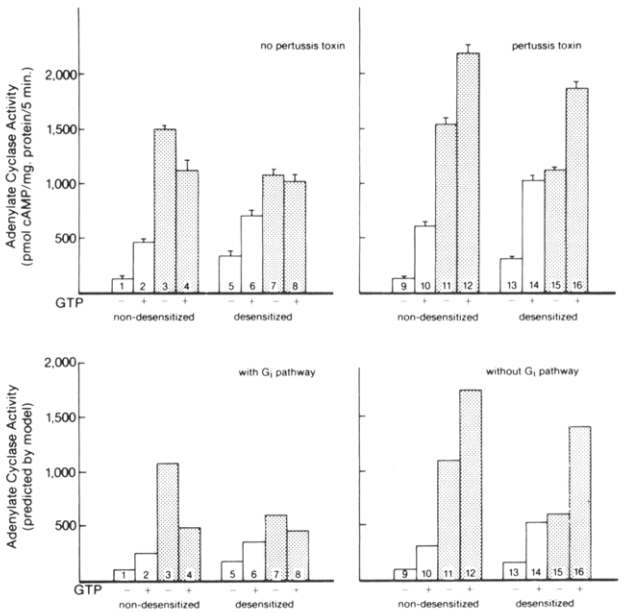

FIGURE 5: Role of G_i in desensitization. The top panel represents the mean ± SEM for four separate experiments. Culture medium was changed without (left) or with (right) pertussis toxin the day prior to assay. Where indicated (desensitized), cells were incubated with LVP (200 nM) at 37 °C for 20 min prior to assay. Basal (clear bars) and 2 μM LVP (shaded bars) stimulated enzyme activities were measured with or without added 100 μM GTP as indicated by the (+) and (–) signs on the horizontal axis. $p < 0.01$ for comparisons: 3 vs. 7, 11 vs. 14, 4 vs. 12, and 8 vs. 16. The bottom panel shows the corresponding model predictions for each incubation condition. Model parameters were the same as those used to generate Table III. K'_G was reduced by 50% to simulate the desensitized state (reduced capacity for hormone to accelerate guanyl nucleotide exchange) as discussed in the text. To account for the experimentally observed residual effect of hormone on basal activity in the desensitized state (approximately 3-fold stimulation), hormone concentration was set at $H = 0.01$.

exchange at G_s were evaluated both in the absence and in the presence of G_i input. As shown in the bottom panel of Figure 5, this resulted in attenuation of hormone-stimulated activity (7 vs. 3), corresponding to experimentally observed desensitization. Furthermore, this attentuation persisted when G_i input was eliminated in the model (15 vs. 11) corresponding to the lack of a pertussis toxin effect on desensitization observed experimentally. The pattern of observed and predicted results was also in agreement with respect to the observed effects of added GTP. With G_i input intact, the observed (top panel) and predicted (bottom panel) results both indicated no increase in enzyme activity with GTP addition (8 vs. 7). With G_i input eliminated, GTP addition was observed (top panel) and predicted (bottom panel) to enhance hormone-stimulated adenylate cyclase activity (16 vs. 15), as it does in nondesensitized cells (12 vs. 11).

Discussion

Tolkovsky et al. (1982) and Blum (1984) have developed and evaluated models for hormonal activation of adenylate cyclase on the basis of the results of radioligand binding and kinetic studies in the intact membrane. These models did not incorporate a possible role for G_i in modulating net activation and did not take into account the subunit structure and dissociation of G_s and G_i, which have only since been appreciated. Previous adenylate cyclase activation models have not accounted for the variable effects of GTP to either augment or inhibit adenylate cyclase activation by a stimulatory hormone in different systems. A biphasic response to GTP for activation of adenylate cyclase by β-adrenergic stimulation was reported

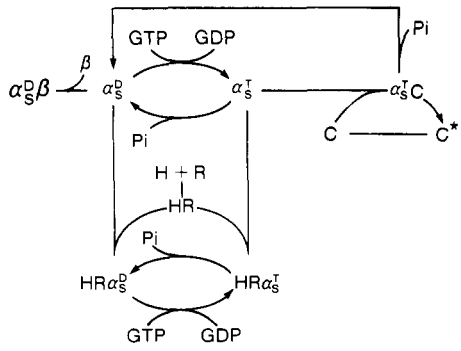

FIGURE 6: Cyclic dissociation model for activation of vasopressin-sensitive adenylate cyclase in $LLCPK_1$ cells. Mathematical formulations with equilibrium and rate constants are given in Verkman et al. (1986), from which this figure is adapted with permission. The superscripts D and T denote the GDP- and GTP-bound states respectively of α_s. The asterisk denotes the activated state of C. Other abbreviations are as outlined in footnote 1.

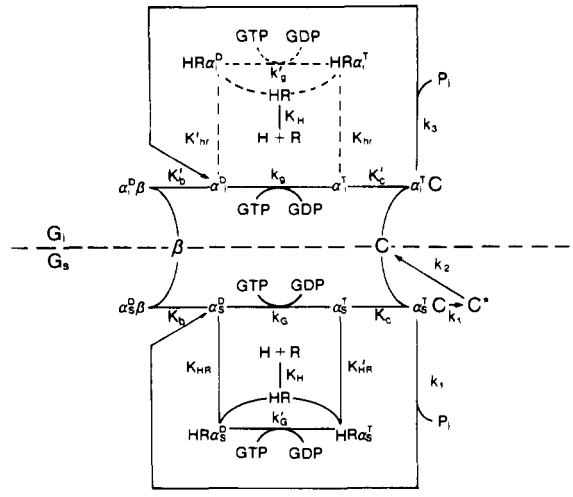

FIGURE 7: Model for cross talk between G_s and G_i in the overall activation of adenylate cyclase. Abbreviations are as for Figure 6 or are given in footnote 1. Reactions shown as broken lines were not required in applying the model to the experimental results. Equilibrium and rate constants are defined in the Appendix. Relative values assigned to these parameters depend on the mechanisms and assumptions of the model and determine steady-state concentrations of the reactants and overall adenylate cyclase activities under varying conditions. Because this formulation represents a minimal model, parameter values should only be used for qualitative descriptions of more complex models of the adenylate cyclase system.

in the rat adipocyte membrane (Cooper et al., 1978; Owens et al., 1985). Low concentrations of GTP resulted in stimulation, and high concentrations of GTP resulted in inhibition of the adenylate cyclase response. Similarly, addition of GTP was noted to inhibit oxytocin-stimulated adenylate cyclase in frog bladder epithelium (Boeckert et al., 1972). These findings contrast with the usual effect of GTP to enhance adenylate cyclase activity by stimulatory ligands (Rodbell et al., 1971; Rodbell, 1980; Gilman, 1984). No overall mechanism has been proposed to explain these varying responses to GTP.

In this study we have demonstrated that addition of GTP inhibits vasopressin-stimulated adenylate cyclase in $LLCPK_1$ membranes. A distinct V_1-receptor subtype did not mediate this inhibitory effect as indicated by use of receptor subtype specific vasopressin analogues. Inhibition was reversed by pretreatment of the cells with pertussis toxin and can be explained by incorporating G_i into the adenylate cyclase activation mechanism. Previously, G_i has been shown to mediate coupling of distinct inhibitory receptors (e.g., opiates, α_2-adrenergic, A_1 adenosine) to adenylate cyclase inhibition. The results of this study indicate that G_i may tonically modulate adenylate cyclase activation in response to stimulatory receptors as well. G_i-mediated inhibition occurred over a GTP concentration range similar to that which supports inhibition of adenylate cyclase by classic inhibitory ligands (Aktories et al., 1983; Seamon & Daly, 1982). The possibility of a tonic modulatory role of G_i for stimulatory receptors is supported by studies in solubilized and reconstituted systems containing purified β-adrenergic receptor, G_s, and G_i (Cerione et al., 1985a,b). These studies demonstrated that addition of G_i was necessary to express the full stimulatory capacity of the β-adrenergic receptor.

We have previously developed a model for the activation of adenylate cyclase by vasopressin, on the basis of radioligand binding studies, enzyme kinetics, and target analysis in $LLCPK_1$ cells (Verkman et al., 1986a,b; Skorecki et al., 1986). According to this model (Figure 6), activation proceeds by the sequential steps of the dissociation of the α_s- and β-subunits of G_s followed by rate-limiting guanyl nucleotide exchange on the dissociated α_s-subunit.[2] This converts α_s from a GDP-bound state to a GTP-bound state, which then interacts with the catalytic unit to form the active moiety whose molecular weight is approximately 180 kDa. Hormonal stimulation accelerates the guanyl nucleotide exchange reaction. Other possible activation mechanisms were not consistent with either the observed enzyme kinetics, radioligand binding data, or radiation inactivation findings (Skorecki et al., 1986). We used the results of this study to incorporate G_i interaction into the overall adenylate cyclase activation mechanism, assuming that activation of G_i also occurs by dissociation of its α_i- and β-subunits and that GTP binds to the α_i-subunit (Gilman, 1984) (Figure 7). This is a general model that includes the possibility of a direct interaction of the receptor with G_i (dashed lines, Figure 7) as is postulated for a classical inhibitory receptor. Both major mechanisms that have been proposed for the inhibitory action of G_i on the basis of studies in solubilized systems are included [mass action effect of β released upon $\alpha_i\beta$ dissociation; direct inhibitory interaction of α_i with the catalytic unit (Gilman, 1984)]. By applying the results of this study to this general model, we examined (1) whether a direct interaction of the receptor with G_i was necessary and (2) whether a mass action of β released upon dissociation of $\alpha_i\beta$ was sufficient to account for GTP-mediated inhibition in the intact membrane system.

We used the results on the left-hand side of Table II to establish the pattern of GTP-modulation of basal and hormone-stimulated enzyme activity. Model parameters were chosen that would match the observed 2–3-fold stimulation of basal activity and 40–50% inhibition of hormone-stimulated activity. As indicated in the Appendix, no direct interaction of the receptor with G_i was required (K_{hr} and $K'_{hr} = \infty$) to generate the results on the left-hand side of Table II with this model. Our ability to find parameters that predict the observed pattern of GTP effects on basal and maximal hormone-stimulated activity was not surprising in itself, due to the large number of parameters contained in the model. Therefore, we used the same parameters to generate the predicted hormone concentration dependency for GTP-mediated inhibition and compared this with corresponding experimental results. Despite the lack of a direct interaction of G_i with the receptor, this model still predicted the inhibitory effect of GTP to be activator concentration dependent with stimulation by added GTP at concentrations of hormone below the $K_{1/2}$ for acti-

[2] For ease of presentation, the γ-subunit is omitted and is assumed to accompany β during dissociation.

vation and inhibition at higher concentrations (Figures 2 and 4). As well, the predicted leftward shift in the $K_{1/2}$ for enzyme activation with GTP was also observed. Therefore, the model provides a mechanism to explain the activator concentration dependent effects of G_i, without requiring its direct interaction with a receptor. This conclusion is further supported by the ability of added GTP to inhibit forskolin-stimulated activity in a manner that is reversed by pertussis toxin (Table IV).

The addition of excess free β in solubilized systems has been shown to inhibit overall adenylate cyclase activity (Northup et al., 1983; Boeckert et al., 1985). This is consistent with a mass action effect of excess free β that would oppose $\alpha_s\beta$ dissociation and is consistent with the models shown in Figures 6 and 7. We evaluated whether such a mass action effect of β released upon $\alpha_i\beta$ dissociation would be sufficent to explain inhibition of adenylate cyclase by added GTP in our system. Despite setting a 5–10-fold stoichiometric excess of G_i over G_s (Codina et al., 1984, Cerione et al. 1985a), reducing the direct inhibitory interaction of α_i with C (increase K'_c) resulted in loss of GTP-mediated inhibition (Table III). While a β mass action effect might account for inhibition by classical inhibitory receptors, or in artificial systems, this is apparently not sufficient to account for the modulatory effect of G_i on activation by stimulatory receptors in the native membrane. Elimination of the interaction of α_i with C also suggested this as a possible locus of action for pertussis toxin, since this resulted in reversal of GTP-mediated inhibition. Eliminating $\alpha_i\beta$ dissociation (decreased K'_b) or decreasing guanyl nucleotide exchange on α_i (decreased k_g) also reversed GTP-mediated inhibition, but they were not as sensitive as changes in K'_c (not shown). Therefore, this model cannot be used to pinpoint a single locus for pertussis toxin action. Of note, in a recent study Huff et al. (1986) have demonstrated persistent $\alpha_i\beta$ subunit dissociation upon stimulation of ADP-ribosylated G_i in a solubilized system. This observation would be consistent with an alteration in K'_c.

Homologous desensitization is receptor-specific. Therefore, lack of a direct interaction of the receptor with G_i would imply that G_i is not involved in homologous desensitization. Indeed, pertussis toxin failed to reverse homologous desensitization in this study (Figure 5). However, with pertussis toxin pretreatment, GTP addition enhanced hormone-stimulated activity in desensitized cells, consistent with a defect in guanyl nucleotide exchange in desensitization. This effect of GTP was not evident unless G_i input was eliminated, because of the concomitant inhibitory effect of G_i at high GTP concentrations. A similar result was reported in cultured renal papillary epithelial cells (Wilson et al., 1986). These studies were done by a microassay for adenylate cyclase in permeabilized cells, without removal of high ambient GTP concentrations. Desensitization was evident in cells not pretreated with pertussis toxin despite the GTP present, as a result of the concomitant effect of GTP to activate G_i. Pertussis toxin eliminated G_i input and resulted in enhanced hormone-stimulated activity, with apparent reversal of desensitization.

The model that has been developed in this study (Figure 7) is based on studies of the vasopressin–adenylate cyclase interaction in renal epithelial membranes. Model development involved finding parameters that could be used to predict the observed effects of added GTP on basal and maximal hormone-stimulated enzyme activity in the absence of pertussis toxin. Model validation in this study involved using these same parameters to generate the predicted hormone concentration dependence for the effects of GTP, the predicted effect of eliminating G_i input, and the predicted effect of densensitization and comparing these with corresponding experimental observations. Further validation will require application to other systems in which there is evidence for G_i input in modulating stimulatory receptor activation of adenylate cyclase.

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Appendix

Model Formulation. A general model for adenylate cyclase activation by stimulatory and inhibitory pathways is shown in Figure 7. Three possible modes of coupling between the pathways have been included: coupling through shared β, C, and R. By varying the parameters describing the inhibitory pathway, the effects of any combination of the coupling pathways can be examined.

The model is described by nine bimolecular equlibrium constants

$$K_b = [\alpha_s{}^D][\beta]/[\alpha_s{}^D\beta] \qquad K'_b = [\alpha_i{}^D][\beta]/[\alpha_i{}^D\beta]$$

$$K_c = [\alpha_s{}^T][C]/[\alpha_s{}^TC] \qquad K'_c = [\alpha_i{}^T][C]/[\alpha_i{}^TC]$$

$$K_{HR} = [\alpha_s{}^D][HR]/[\alpha_s{}^DHR]$$

$$K'_{HR} = [\alpha_s{}^T][HR]/[\alpha_i{}^THR]$$

$$K_{hr} = [\alpha_i{}^D][HR]/[\alpha_i{}^DHR]$$

$$K'_{hr} = [\alpha_i{}^T][HR]/[\alpha_i{}^THR]$$

$$K_H = [H][R]/[HR] \qquad \text{(A1)}$$

three steady-state rate equations

$$0 = d[C^*]/dt = k_1[\alpha_s{}^TC] - k_2[C^*]$$

$$0 = d[\alpha_s{}^D]/dt = k_1[\alpha_s{}^TC] - k_G[\alpha_s{}^D] - k'_G[\alpha_s{}^DHR]$$

$$0 = d[\alpha_i{}^D]/dt = k_3[\alpha_i{}^TC] - k_g[\alpha_i{}^D] - k'_g[\alpha_i{}^DHR] \qquad \text{(A2)}$$

and five conservation conditions in which total subunit stoichiometries, $[\alpha_i]_T$, $[\alpha_s]_T$, $[\beta]_T$, $[C]_T$, and $[R]_T$, are expressed as the sum of concentrations of the appropriate reaction components.

The 17 equations in 17 unknowns can be cast into a set of five nonlinear coupled algebraic equations:

$$[\alpha_s{}^D] = [\alpha_s]_T/(1 + [\beta]/k_b + [H][R]/(K_HK_{HR}) + X_1[1 + [C]/K_c + [H][R]/(K_HK'_{HR})])$$

$$[\alpha_i{}^D] = [\alpha_i]_T/(1 + [\beta]/K'_b + [H][R]/(K_HK_{hr}) + X_2[1 + [C]/K'_c + [H][R]/(K_HK'_{hr})])$$

$$[\beta] = [\beta]_T/(1 + [\alpha_i{}^D]/K'_b + [\alpha_s{}^D]/K_b)$$

$$[C] = [C]_T/[1 + X_1[\alpha_s{}^D](k_1/k_2 + 1)/K_c + X_2[\alpha_i{}^D]/K'_c]$$

$$[R] = [R]_T/[([H]/K_H)(1 + [\alpha_s{}^D]/K_{HR} + [\alpha_s{}^D]X_1/K'_{HR} + [\alpha_i{}^D]/K_{hr} + [\alpha_i{}^D]X_2/K'_{hr})] \qquad \text{(A3)}$$

where

$$X_1 = K_c[k_G + k'_G[H][R]/(k_1K_HK_{HR}[C])]$$

$$X_2 = K'_c[k_g + k'_g[H][R]/(k_3K_HK_{HR}[C])] \qquad \text{(A4)}$$

Equation A3 was solved iteratively using the following initial concentrations:

$$[\alpha_s{}^D] = [\alpha_s]_T/2 \qquad [\alpha_i{}^D] = [\alpha_i]_T/2 \qquad [\beta] = [\beta]_T/2$$

$$[C] = [C]_T/2 \qquad [R] = [R]_T/2$$

New concentrations on the left-hand side of the equation were calculated by using old concentrations from the previous iteration. The procedure was continued until all concentrations changed by less than 0.01%. The calculation was performed on an IBM/XT computer in BASIC 3.0 compiled by an 8087/INLINE compiler (Microway Inc., Bedford, MA). For any set of parameters, the steady-state concentrations of the reactants were calculated and tabulated in under 5 s. For the parameters used in Table III, the calculated steady-state concentrations of the reactants in the –GTP, basal/VP state were as follows: α_s^D, 0.52/0.36; α_i^D, 0.93/0.88; β, 0.92/0.98; C, (0.48/3.4) × 10^{-4}, R, 1.0/0.00072; HR, 0/0.72; α_s^T, (1.3 × 10^{-6}/0.16; α_i^T, (1.0 × 10^{-5})/0.013; HRα_s^D, 0/0.26; HRα_s^T, 010/018; $\alpha_i^D\beta$, 8.6/8.7; $\alpha^{sD}\beta$, 0.48/0.35; α_i^TC, 0.47/0.44; α_s^TC, (5.2 × 10^{-4})/0.0055; C*, 0.052/0.55.

In addition, a program was developed to plot the expected adenylate cyclase activity and steady-state concentrations of the reactants for continuous variations of any parameter. The programs are available upon request from the authors.

Model Application. The model was used to examine the mechanism of coupling between the stimulatory and inhibitory pathways and the mechanism of desensitization based on data given in Table II. We first determined the simplest form of the model that could account for the data on the left side of Table II (absence of pertussis toxin). Parameters used for the stimulatory pathway were taken from previous target analysis experiments (Skorecki et al., 1986). Parameters for the inhibitory pathway were varied systematically to obtain agreement between the pattern of calculated and experimental results. Predicted activities were most sensitive to changes in any of the rate constants, as well as to changes in K_b, K'_b, K_C, and K'_c. A change of 50% in these parameters resulted in the loss of predicted order of activities shown in Table III (VP and –GTP > VP and +GTP > basal and +GTP > basal and –GTP). The actual parameters used are listed in Table III.

If the only mode of coupling were through β (i.e., $k'_g = 0$ and $K_{hr} = K'_{hr} = K'_c = \infty$), no values for $[\alpha_i]_T$, K_b, and K_g were found in which the pattern of calculated results was similar to that of the experimental findings. Only inclusion of the α_i^TC binding reaction allowed both stimulation of basal activity and inhibition of hormone-stimulated activity by added GTP and yielded the results in the left panel of Table III. These results were obtained without requiring any interaction between R and the inhibitory pathway ($k'_g = 0$; $K_{hr} = K'_{hr} = \infty$) and indicate that a minimal model need not postulate a direct interaction between R and G_i. We further determined whether this minimal model that satisfied the effects of GTP on basal and maximal hormone-stimulated activity would also yield the patterns of experimental results obtained with pertussis toxin with varying hormone concentration, and for desensitization. The pattern of results obtained experimentally with pertussis toxin is shown in Table II (little effect on basal activity or on hormone-stimulated activity in the absence of added GTP, enhancement of hormone-stimulated activity in the presence of added GTP) and was obtained for the model by eliminating interaction of the G_i pathway with G_s (decreasing K'_b or K_g or increasing K'_c). K'_c was the most sensitive parameter, and the results shown in Table III are for a 100-fold increase in K'_c (parameter values given in Table III). The predicted results for varying H/K_H are shown in Figure 4. The parameter changes used and calculated results obtained for desensitization are shown in Figure 5.

Registry No. LVP, 50-57-7; GTP, 86-01-1; dDAVP, 16679-58-6; adenylate cyclase, 9012-42-4; forskolin, 66575-29-9.

References

Aktories, K., Schultz, G., & Jakobs, K. H. (1983) *Mol. Pharmacol. 24*, 183–188.

Blum, J. J. (1984) *J. Theor. Biol. 111*, 589–608.

Boeckert, J., Roy, C., & Jard, S. (1972) *J. Biol. Chem. 247*, 7073–7081.

Boeckert, J., Deterre, P., Pfister, C., Guillon, G., & Chabre, M. (1985) *EMBO J. 4*, 1413–1417.

Cerione, R. A., Codina, J., Kilpatrick, B. F., Staniszewski, C., Gierschik, P., Somers, R. L., Spiegel, A. M., Birnbaumer, L., Caron, M. G., & Lefkowitz, R. J. (1985a) *Biochemistry 24*, 4499–4503.

Cerione, R. A., Staniszewski, C., Caron, M. G., Lefkowitz, R. J., Codina, J., & Birnbaumer, L. (1985b) *Nature (London) 318*, 293–295.

Codina, J., Hildebrandt, J. D., Sekura, R. D., Birnbaumer, M., Bryan, J., Manclark, R., Iyengar, R., & Birnbaumer, L. (1984) *J. Biol. Chem. 260*, 5871–5866.

Cooper, D. M. F., Schlegel, W., Lin, M. C., & Rodbell, M. (1979) *J. Biol. Chem. 254*, 8927–8931.

Gilman, A. G. (1984) *Cell (Cambridge, Mass.) 36*, 577–579.

Goldring, S. R., Dayer, J. M., Ausiello, D. A., & Krane, S. M. (1978) *Biochem. Biophys. Res. Commun. 83*, 434–440.

Huff, R. M., & Neer, E. J. (1986) *J. Biol. Chem. 261*, 1105–1110.

Hull, R. N., Cherry, W. R., & Weaver, G. W. (1976) *In Vitro 12*, 670–677.

Katada, T., & Ui, M. (1982a) *J. Biol. Chem. 257*, 7210–7216.

Katada, T., & Ui, M. (1982b) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3129–3133.

MacFarlane, D. E. (1982) *Mol. Pharmacol. 22*, 580–588.

Northup, J. K., Sternweiss, P. C., & Gilman, A. G. (1983) *J. Biol. Chem. 258*, 11361–11368.

Owens, J. R., Frame, L. J., Ui, M., & Cooper, D. M. F. (1985) *J. Biol. Chem. 260*, 15946–15952.

Rodbell, M. (1980) *Nature (London) 284*, 17–22.

Rodbell, M., Birnbaumer, L., Pohl, S. L., & Krans, H. M. J. (1971) *J. Biol. Chem. 246*, 1877–1882.

Roy, C., Hall, D., Karish, M., & Ausiello, D. A. (1981) *J. Biol. Chem. 256*, 3424–3427.

Salomon, Y., Lapidot, M., & Ezra, E. (1984) *Ann. Endocrinol. 45*, 327–334.

Seamon, K. B., & Daly, J. W. (1982) *J. Biol. Chem. 257*, 11591–11596.

Skorecki, K. L., Verkman, A. S., Jung, C. Y., & Ausiello, D. A. (1986) *Am. J. Physiol. 250*, C115–C123.

Tolkovsky, A. M., Braun, S., & Levitski, A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 213–217.

Verkman, A. S., Skorecki, K. L., & Ausiello, D. A. (1986a) *Am. J. Physiol. 250*, C103–C114.

Verkman, A. S., Ausiello, D. A., Jung, C. Y., & Skorecki, K. L. (1986b) *Biochemistry 25*, 4566–4572.

Wilson, P. D., Dixon, B. S., Dillingham, M. A., Garcia-Sainz, J. A., & Anderson, R. J. (1986) *J. Biol. Chem. 261*, 1503–1506.