Nucl. Instrum. Methods Phys. Res. 172, 195–199.
Sutherland, J. C., Keck, P. C., Griffin, K. P., & Takacs, P. Z. (1982) *Nucl. Instrum. Methods Phys. Res. 195*, 375–379.
Thornber, J. P., & Thornber, J. M. (1980) in *Photosynthesis and Nitrogen Fixation* (San Pietro, A., Ed.), pp 172–178, Academic Press, New York.
Thornber, J. P., Seftor, R. E. B., & Cogdell, R. J. (1981) *FEBS Lett. 134*, 235–239.
Vermeglio, A., & Paillotin, G. (1982) *Biochim. Biophys. Acta 681*, 32–40.
Wasielewski, M. R., Smith, V. H., & Norris, J. R. (1982) *FEBS Lett. 149*, 138–140.

Transducin and the Inhibitory Nucleotide Regulatory Protein Inhibit the Stimulatory Nucleotide Regulatory Protein Mediated Stimulation of Adenylate Cyclase in Phospholipid Vesicle Systems†

Richard A. Cerione,*,‡ Juan Codina,⊥ Brian F. Kilpatrick,‡,§ Claudia Staniszewski,‡ Peter Gierschik,∥ Robert L. Somers,∥ Allen M. Spiegel,∥ Lutz Birnbaumer,⊥ Marc G. Caron,‡ and Robert J. Lefkowitz‡

Howard Hughes Medical Institute and Departments of Medicine, Biochemistry, and Physiology, Duke University Medical Center, Durham, North Carolina 27710, National Institutes of Health, NIAMDD, Bethesda, Maryland 20205, and Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

Received May 2, 1985

ABSTRACT: The adenylate cyclase coupled inhibitory nucleotide regulatory protein (N_i) and the bovine retinal nucleotide regulatory protein transducin (T) appear to share some common functional properties since their GTPase activity is stimulated to similar extents by the retinal photoreceptor rhodopsin. In the present work, we sought to assess whether these functional similarities might extend to their interaction with adenylate cyclase. This necessitated the development of reconstitution systems in which guanine nucleotide regulatory protein mediated inhibition of adenylate cyclase activity could be demonstrated and characterized in a lipid milieu. In the absence of the pure human erythrocyte stimulatory nucleotide regulatory protein (N_s), the insertion into phospholipid vesicles of either pure N_i from human erythrocytes or pure bovine T with the resolved catalytic moiety of bovine caudate adenylate cyclase (C) does not establish GppNHp inhibition of either Mg^{2+}- or forskolin-stimulated adenylate cyclase. However, the coinsertion into lipid vesicles of either N_i or T with N_s and resolved C results in an inhibition of N_s(GppNHp) stimulatable C activity. As is the case in intact membranes, the reconstituted inhibition of the N_s-stimulated C activity extends into the steady-state phase of time courses of activity. This inhibition is highly sensitive to the $MgCl_2$ concentration. At 2 mM $MgCl_2$, the inhibition is greater than 80% while at 50 mM $MgCl_2$ it is only ~20%. Overall these results suggest (1) that the inhibition of adenylate cyclase activity by N_i is due to an interference with the N_s stimulation of the C activity rather than a direct effect on the intrinsic activity of the catalytic moiety itself and (2) that the retinal nucleotide regulatory protein, transducin, substitutes for N_i in causing inhibition of N_s-stimulated C activity.

Adenylate cyclase activity is regulated through distinct stimulatory and inhibitory pathways. Stimulation is initiated by a hormone–receptor (β-adrenergic, glucagon, prostaglandin E_1, etc.) interaction which results in the promotion of GTP binding to the stimulatory nucleotide binding regulatory protein (N_s). The N_s·GTP complex then directly interacts with the catalytic moiety of adenylate cyclase (C). This protein has been purified to homogeneity from several sources (Northup et al., 1980; Sternweis et al., 1981; Codina et al., 1984; Hanski et al., 1981, 1982) and has been found to be heterotrimeric (molecular weight: α, 42000–52000; β, 35000; γ, ~5000) (Gilman, 1984; Hildebrandt et al., 1984a).

In a similar manner, the inhibition of adenylate cyclase activity is initiated by a hormone–receptor (α_2-adrenergic, muscarinic, dopamine, etc.) interaction which effects the activation of an inhibitory nucleotide binding regulatory protein (N_i). This regulatory protein has recently been purified to apparent homogeneity, and like N_s, it is heterotrimeric (molecular weight: α, 39000–41000; β, 35000; γ, ~5000) (Codina et al., 1983, 1984; Bokoch et al., 1983).

A significant amount of information regarding the activation of N_s and its resultant stimulation of adenylate cyclase activity has been obtained both from studies in intact membranes (Iyengar & Birnbaumer, 1980; Northup et al., 1983) and more recently from reconstituted systems (Asano et al., 1984; Asano & Ross, 1984; Cerione et al., 1984, 1985). Recently, attention has begun to focus on understanding the manner in which activated N_i inhibits C activity. Various mechanisms have been postulated including the $\beta\gamma$-deactivation hypothesis (Gilman, 1984; Katada et al., 1984). This model suggests that the activation of nucleotide regulatory proteins reflects subunit dissociation such that upon activating N_i, the increased levels of $\beta\gamma$ can interfere with the activation (and subunit dissoci-

† This work was supported in part by National Institutes of Health Research Grant AM-19318 to L.B., National Institutes of Health Research Grant HL-16037 to R.J.L., and Deutsche Forschungsgemeinschaft Grant Gi 138/1-1 to P.G. J.C. is a trainee of NIH Diabetes and Endocrinology Training Grant AM-07348.

* Address correspondence to this author at Howard Hughes Medical Institute, Duke University Medical Center.
‡ Duke University Medical Center.
⊥ Baylor College of Medicine.
§ Present address: A. H. Robins Co., Research Laboratories, Richmond, VA 23220.
∥ National Institutes of Health.

0006-2960/85/0424-4499$01.50/0

ation) of N_s. However, not all results are consistent with the inhibition being due to a simple $\beta\gamma$-deactivation of N_s, and in fact, some findings suggest that N_i, like N_s, can directly interact with the catalytic moiety (Hildebrandt et al., 1983, 1984b; Katada et al., 1984). Thus, the predominant mechanism(s) operating in the inhibition of C activity still remain(s) to be determined.

The goal of the present studies was to develop a reconstitution system, analogous to those used to study activation of C by N_s (Cerione et al., 1984), for investigating the interaction of N_i with the adenylate cyclase system. We also wished to investigate the effect, if any, of the retinal guanine nucleotide regulatory protein transducin (T) on the adenylate cyclase. Although previously reported to stimulate the enzyme (Bitensky et al., 1982), our recent results have suggested a much stronger analogy of this protein to N_i than N_s. Thus, both T and N_i are activated by rhodopsin while N_s is not, and the β-adrenergic receptor activates N_s but only weakly interacts with N_i and T (Cerione et al., 1985). Our results not only shed light on the mechanisms of N_i-mediated inhibition of enzyme activity but also add further evidence for the marked functional homology of N_i and T.

Experimental Procedures

Materials. Octyl β-D-glucopyranoside (octyl glucoside) was obtained from Calbiochem, Extracti-gel D was from Pierce Chemical Co., 5′-guanylyl imidodiphosphate (GppNHp) was from Boehringer Mannheim, and [α-^{32}P]ATP was from New England Nuclear. All other materials were from sources previously described (Cerione et al., 1984, 1985).

Preparation of the Various Protein Components. Both N_s and N_i were solubilized from human erythrocytes by using sodium cholate and then purified (>90%) as intact heterotrimeric holoproteins (α, β, γ) as described (Codina et al., 1984). These preparations were stored at −90 °C in 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 20 mM β-mercaptoethanol, 30% ethylene glycol, 150 mM NaCl, and 50 μg/mL bovine serum albumin and in 1.65–7% Lubrol PX.

The catalytic moiety (C) of adenylate cyclase was solubilized from bovine caudate nucleus with sodium cholate and isolated from the other components of the system by Sepharose 6B chromatography as described by Strittmatter & Neer (1980). The adenylate cyclase preparations were typically stored in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 200 mM sucrose, 1 mM dithiothreitol (DTT), 15 mM $MgCl_2$, 3.5 mg/mL crude soybean phosphatidylcholine, and 0.6% sodium cholate, pH 7.6, or in some instances in 25 mM Tris-HCl, 100 mM NaCl, 1 mM DTT, 2 mM $MgCl_2$, 1 mM EDTA, 0.1% bovine serum albumin, and 0.2% sodium cholate, pH 7.6.

The rhodopsin was purified (>90%) from a 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) extract of bovine retinal rod outer segments, as previously described (Litman, 1982), and then stored at −90 °C in 10 mM CHAPS, 50 mM Tris–acetate, 0.1 M methyl α-mannoside, 0.1 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, pH 7.0. The transducin was purified (>90%) as an intact heterotrimeric holoprotein (α, β, γ) essentially as described by Baehr et al. (1982) and was stored at −90 °C in 50% glycerol, 10 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM dithiothreitol, pH 7.5.

Insertion of Protein Components in Phospholipid Vesicles. The insertion of the different nucleotide regulatory proteins and C preparations into phospholipid vesicles was performed as follows. Soybean phosphatidylcholine (0.1 mL of a 17 mg/mL sonicated solution) was incubated with 0.05–0.15 mL of the adenylate cyclase preparation (typically yielding 1.25 nmol of forskolin-stimulated cAMP production per 30 min at 30 °C), 0.19–0.28 mL of 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), and 25 μL of octyl glucoside (17%) for 20–30 min on ice. At this point, the nucleotide regulatory proteins (~0.5 μg of N_s in 5–15 μL; 1.25 μg of N_i in 25 μL; 1.47 μg of transducin in 3 μL) and rhodopsin (6.8 μg in 15 μL) were added to the lipid solutions (incubated ~1–2 min at 4 °C). Bovine serum albumin (10 μL of 50 mg/mL) was also included in the incubations with the final volume being 0.5 mL. These mixtures were then immediately applied to Extracti-gel columns (1 mL of gel) at 4 °C which were pretreated with 4 volumes of 100 mM NaCl and 10 mM Tris-HCl (pH 7.4) containing 2 mg/mL BSA and then equilibrated with 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), and 1 mM dithiothreitol. Eluates from the Extracti-gel columns (2 mL in the above dithiothreitol-containing buffer) were incubated with 0.6 mL of 50% poly(ethylene glycol) (M_r 6000–8000) for 5 min at room temperature, then diluted with 16 mL of 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), and 0.16 mL of dithiothreitol (0.1 M), and centrifuged at 250000*g* for 1.5 h at 4 °C. The resultant protein–lipid pellets were resuspended in 75 mM Tris-HCl (pH 7.8) and 1 mM dithiothreitol (final volume 0.30–0.85 mL) and assayed for adenylate cyclase activity as described below. The amounts of N_s and N_i are expressed in terms of total protein, determined as previously described (Codina et al., 1984); molar concentrations of these proteins were determined by assuming a molecular weight of 95 000 (Codina et al., 1984). The amounts of transducin are expressed in terms of total protein as determined by the method of Lowry et al. (1951); molar concentrations of rhodopsin were calculated by using an extinction coefficient for rhodopsin of 4.06×10^4 M^{-1} at 498 nm (Wald & Brown, 1983). The efficiencies of reconstitution of the different protein components range from 20% to 50% (Cerione et al., 1984, 1985).

Adenylate Cyclase Assays. The adenylate cyclase assays were performed in a total volume of 0.1 mL containing 50 mM NaHEPES (pH 8.0), 1 mM EDTA, 0.25 mM ATP, 3 mM phosphoenolpyruvate, 10 μg/mL pyruvate kinase, 0.1 mg/mL bovine serum albumin, 0.1 mM cAMP, 10 μg/mL myokinase, 2 μCi of [α-^{32}P]ATP, and varying concentrations of $MgCl_2$ as indicated. The enzyme activity was assayed for 30 min at 30 °C (unless otherwise indicated) as described by Salomon et al. (1974). The results presented below represent the means of duplicate/triplicate determinations, the ranges of which were usually within 10%, from a single experiment (repeated at least twice, unless otherwise noted).

Results

Effects of N_s, N_i, and Transducin (T) on the Activity of Adenylate Cyclase (C). The catalytic moiety of adenylate cyclase (C) from bovine caudate, which is resolved from the other components of the system by Sepharose 6B chromatography, can be inserted into phosphatidylcholine vesicles with the aid of octyl glucoside (Cerione et al., 1984). These vesicles, in the presence of $MgCl_2$, yield a basal level of cyclic AMP production which is essentially unaffected by the addition of guanine nucleotides (Cerione et al., 1984). However, upon the coinsertion of pure human erythrocyte N_s together with the resolved C, the activity becomes responsive to GppNHp (Cerione et al., 1984). The coinsertion of pure N_s with C also effects an increase in the forskolin-stimulatable C activity, and this activity is stimulated still further upon the addition of GppNHp (Figure 1A). These results document the recon-

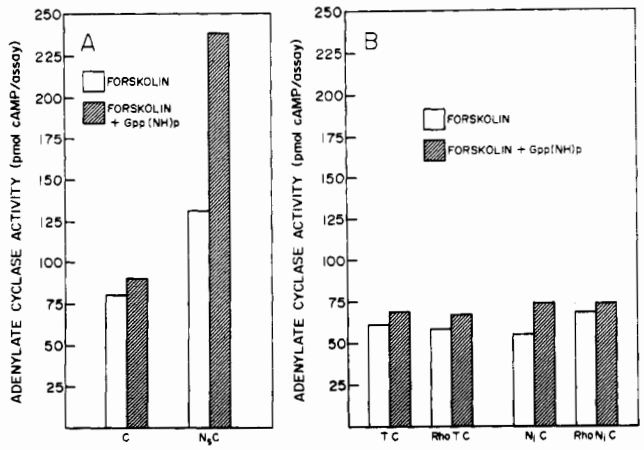

FIGURE 1: Forskolin-stimulatable adenylate cyclase activity in phospholipid vesicles containing resolved C and various pure guanine nucleotide regulatory proteins. Resolved C preparation (100 μL), 0.49 μg of N_s ($\simeq$5 pmol), 1.25 μg of N_i ($\simeq$13 pmol), 1.47 μg of transducin ($\simeq$15 pmol), and 6.8 μg of rhodopsin (rho) ($\simeq$195 pmol) were added to reconstitution incubations as described under Experimental Procedures. The phospholipid vesicles were suspended in 0.35 mL of 75 mM Tris-HCl, pH 7.8, and 1 mM DTT, and adenylate cyclase activity was determined on 20-μL aliquots of the resuspended vesicles in a final assay volume of 50 μL ([$MgCl_2$] = 10 mM). (A) Responsiveness of vesicles containing resolved C alone, or resolved C and pure N_s, to 10^{-4} M forskolin or to 10^{-4} M forskolin plus 10^{-4} M GppNHp. (B) Responsiveness of vesicles containing resolved C and pure N_i, or pure T, to 10^{-4} M forskolin or to 10^{-4} M forskolin plus 10^{-4} M GppNHp. The results shown are representative of two experiments.

stitution of functional interactions between the N_s and C components in these lipid vesicles. The very slight increase in the forskolin-stimulatable activity, which is obtained upon the addition of GppNHp to vesicles containing C alone, suggests that the C preparations used in these experiments may still contain small quantities of N_s.

The results of the coinsertion of the pure human erythrocyte N_i with the resolved C into phosphatidylcholine vesicles are presented in Figure 1B. (In these experiments, [N_i] was $\sim$2.5-fold greater than the levels of N_s used in the above studies.) In the presence of forskolin, the addition of GppNHp to the vesicles containing N_i and C does not inhibit enzyme activity, when compared with the activity observed in the absence of added guanine nucleotide, and in fact causes slight increases in activity (Figure 1B). Similarly, the addition of GppNHp to vesicles containing N_i and C causes no inhibition of the basal ($MgCl_2$-stimulatable) C activity, compared to the activity obtained in vesicles containing C alone (data not shown). To ensure the complete activation of the N_i in these vesicles, experiments were performed in which light-activated rhodopsin was coinserted with N_i and C into phosphatidylcholine vesicles. Previously, we and others have demonstrated that light-activated rhodopsin can effectively activate N_i, as monitored by stimulation of GTPase activity, in phosphatidylcholine vesicles (Kanaho et al., 1984; Cerione et al., 1985). However, as shown in Figure 1B, the presence of rhodopsin does not modify the lack of a GppNHp-mediated inhibition by N_i on these activities.

Figure 1B shows that very similar results are obtained when the guanine nucleotide regulatory protein from the bovine retinal system, transducin (T), is coinserted with the caudate C into lipid vesicles. The addition of GppNHp to these vesicles (in either the presence or the absence of light-activated rhodopsin) has little or no effect on the adenylate cyclase activity and again causes a slight stimulation of the forskolin-stimulatable activity, just as was the case for vesicles containing the pure N_i. Taken together, the results presented in Figure 1 clearly demonstrate the lack of any direct (GppNHp-sensitive) inhibition of the intrinsic C activity by N_i or T under these experimental conditions.

Effects of N_i, or Transducin (T), on the N_s(GppNHp)-Stimulated Adenylate Cyclase (C) Activity. While neither pure N_i nor pure T has any direct effects on the intrinsic activity of C in the phosphatidylcholine vesicles, both of these proteins show significant inhibition of N_s-stimulated adenylate cyclase activity. Specifically, when N_s and C have been coinserted into lipid vesicles with pure N_i or pure T [where [N_i] $\simeq$ [T] $\simeq$ (2.5–3)[N_s]], T effects a 35 $\pm$ 3% (SE, n = 5) inhibition of the activity in the presence of GppNHp, while N_i causes a 46 $\pm$ 5% (SE, n = 6) inhibition of the activity in the presence of GppNHp. Under these conditions, the coinsertion of light-activated rhodopsin has little or no effect on the inhibitions by N_i and T (data not shown) since both of these nucleotide regulatory proteins are effectively activated at the $MgCl_2$ (10 mM) and GppNHp (10^{-4} M) concentrations used in these experiments.

The inhibitory actions of N_i and T are not the result of any nonspecific effects resulting from buffer components since the storage buffer components for these two regulatory proteins are completely different (Experimental Procedures); in addition, all of the buffer components are also included in the vesicles containing just N_s and C. Various control experiments also indicate that the above inhibitory effects are not due to N_i or T interfering with the insertion of N_s into lipid vesicles. Specifically, experiments were performed where N_s (5 pmol) was added to one reconstitution incubation, N_i (13 pmol) was added to a second incubation, and both N_s (5 pmol) and N_i (13 pmol) were added together to a third incubation (these latter conditions being similar to those for which N_i inhibition of N_s-stimulated C activity is observed). Incorporation of the nucleotide regulatory proteins into lipid vesicles was then assessed by performing ^{35}S-labeled guanosine 5′-*O*-(3-thiotriphosphate) ([^{35}S]GTPγS) binding (at 3 μM [^{35}S]GTPγS and 50 mM $MgCl_2$; Cerione et al., 1985) on the isolated vesicles. Typical results from such a control experiment were as follows: 0.60 pmol of N_s incorporated into the vesicles prepared from the first incubation, 2.0 pmol of N_i incorporated into the vesicles from the second incubation, and 2.70 pmol of total nucleotide regulatory protein (N_s + N_i) incorporated into the vesicles obtained from the third incubation. The fact that the total nucleotide regulatory protein which is incorporated into the latter vesicles is essentially identical with the arithmetic sum of the individual insertions of N_s and N_i clearly indicates that N_i is not interfering with the insertion of N_s. Moreover, preparations of T may be directly added to phospholipid vesicles which *already contain* N_s and resolved C, since T is prepared in the absence of detergent (Experimental Procedures). When this is done, the levels of inhibition by T are essentially identical with those reported above. This further indicates that the inhibition cannot be explained by a reduced insertion of N_s, and we thus conclude that these results document the reconstitution of inhibition of the N_s-stimulated C activity by N_i or T.

The time courses for the net GppNHp-stimulatable C activities in vesicles containing N_s and C or N_s, N_i, and C at two different $MgCl_2$ concentrations (5 and 20 mM) are shown in Figure 2. As has been well documented in studies on intact membranes (Iyengar & Birnbaumer, 1980), the GppNHp stimulation of the C activity shows an initial lag (presumably reflecting the initial activation of N_s) prior to reaching a steady-state situation. The inhibition by N_i appears to persist

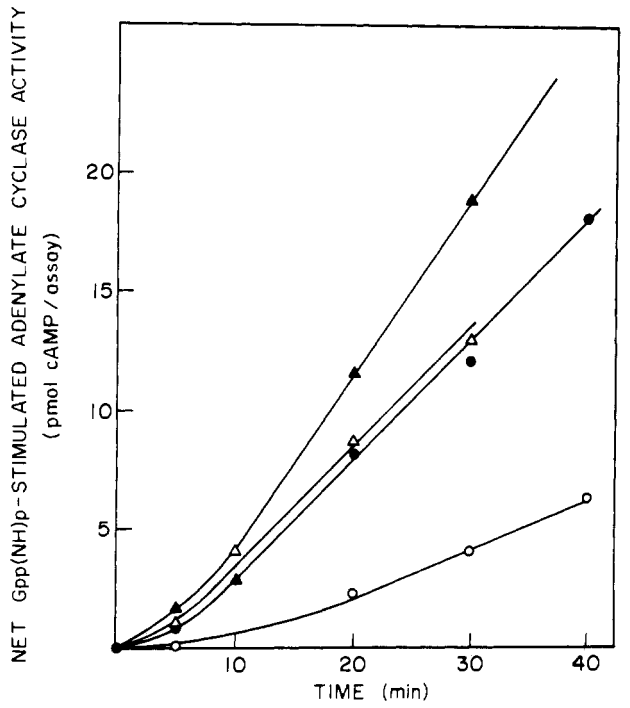

FIGURE 2: Time courses for N_s-mediated GppNHp-stimulated C activities. Resolved C preparation (50 μL) and 0.49 μg of N_s ($\simeq$5 pmol) were added to reconstitution incubations in the presence (△, ○) and absence (▲, ●) of 1.25 μg of N_i ($\simeq$13 pmol) as described under Experimental Procedures. The phospholipid vesicles were resuspended in 0.85 mL of 75 mM Tris-HCl, pH 7.8, and 1 mM DTT, and adenylate cyclase activity was determined as described under Experimental Procedures at either 5 mM $MgCl_2$ (●, ○) or 20 mM $MgCl_2$ (▲, △). Each data point represents the mean of triplicate determinations. The activity presented represents the total activity minus the basal activity (i.e., activity in the absence of added GppNHp).

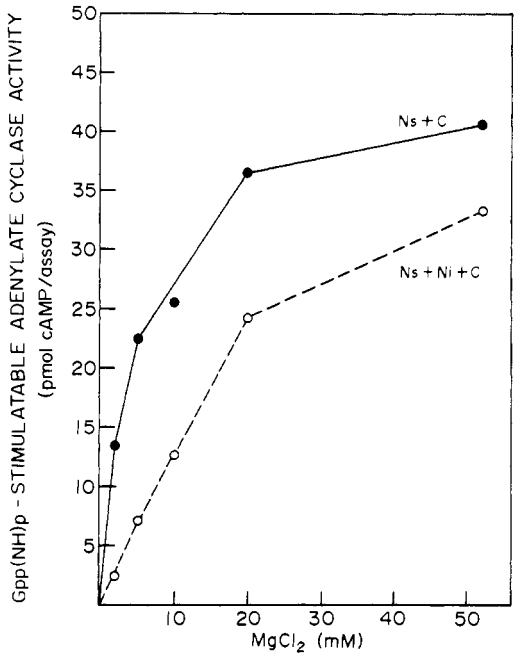

FIGURE 3: Magnesium concentration dependence of the N_i-mediated inhibition of N_s(GppNHp)-stimulatable C activity. Resolved C preparation (50 μL) and 0.49 μg of N_s ($\simeq$5 pmol) were added to the reconstitution incubations in the presence (○) and absence (●) of 1.25 μg of N_i ($\simeq$13 pmol) as described under Experimental Procedures. The phospholipid vesicles were resuspended in 0.8 mL of 75 mM Tris-HCl, pH 7.8, and 1 mM DTT, and adenylate cyclase activity was determined on 20-μL aliquots of the resuspended vesicles in a final assay volume of 50 μL. [GppNHp] = 10^{-4} M. Each data point represents the mean of triplicate determinations from a single experiment which was replicated twice with comparable results.

beyond the initial activation of N_s into the steady-state phase of the activity, with the extent of the inhibition being dependent on the concentration of $MgCl_2$. Further support for the latter point is presented in Figure 3, where it is clear that the N_i inhibition of N_s(GppNHp)-stimulatable adenylate cyclase activity can be dramatically altered in the same vesicle preparations by altering [$MgCl_2$]. Under these conditions, at low [$MgCl_2$], the inhibition by N_i is quite dramatic (~82%) while at 50 mM $MgCl_2$ this inhibition is significantly reduced (~18%). Essentially identical results are obtained when transducin is substituted for N_i in these vesicle systems (data not shown).

Discussion

One of the aims of the present studies was to reconstitute the effects of the inhibitory nucleotide binding regulatory protein (N_i) on the C activity in phospholipid vesicles. Such a system should ultimately provide a means for the delineation of the specific mechanism(s) responsible for N_i-mediated inhibition of C activity in a lipid milieu under conditions which mimic a membrane environment. In addition, we were also interested in determining the potential effects of the analogous nucleotide regulatory protein from the visual transduction system, transducin, on the adenylate cyclase activity in these systems.

We chose to begin these studies with rather simple phospholipid vesicle systems containing C alone or C coinserted with the different nucleotide regulatory proteins (N_s, N_i, and T). The presence of N_s in lipid vesicles containing C results in a marked stimulation by GppNHp of the Mg^{2+}- and forskolin-induced C activities. The coinsertion of N_i, or T, with C, however, does not cause a corresponding inhibition of these C activities and in fact causes a slight GppNHp-dependent stimulation of the forskolin-induced activity. The latter may reflect some form of interaction between N_i (or T) and C which results in a slight stabilizing effect.

The above results contrast with those obtained where N_i, or T, has been coinserted with both N_s and C into lipid vesicles. In these cases, either N_i or T causes a significant inhibition of the N_s(GppNHp)-stimulatable C activities. Thus, these studies clearly indicate that N_i exerts its inhibitory action primarily by affecting the direct N_s stimulation of the bovine caudate C activity rather than through a direct modulation of the intrinsic C activity. The conclusions we reach from our studies differ from those derived from various studies using the cyc$^-$ mutant of the S49 lymphoma cell line. In these mutant cells, guanine nucleotide dependent inhibition by N_i is observed in the absence of functional N_s (Hildebrandt et al., 1982; Katada et al., 1984). The lack of any direct inhibition by N_i or T on the bovine caudate C activity might reflect either differences in the catalytic properties of the caudate and S49 adenylate cyclases or differences in the mechanisms by which N_i mediates inhibition in these two systems.

The fact that N_i and transducin show very similar effects in this reconstituted adenylate cyclase system supports earlier work in two-component (i.e., light-activated rhodopsin and N_i, or transducin) reconstituted systems where it was observed that rhodopsin induces essentially the same total GTPase activities in these two nucleotide regulatory proteins (Kanaho et al., 1984; Cerione et al., 1985). However, it appears to contradict an earlier report (Bitensky et al., 1982) that transducin stimulates C activity.

Overall, these findings have interesting implications regarding the mechanism(s) of hormone-induced inhibition of C activity. Since N_i and transducin appear to share an identical β subunit (Gilman, 1984), the results presented here are consistent with the β subunit having an important role in the inhibitory process (Gilman, 1984; Katada et al., 1984). Nevertheless, the apparent functional similarities exhibited by the α subunits of these two regulatory proteins, as indicated in GTPase experiments (Kanaho et al., 1984; Cerione et al., 1985), also leave open the possibility of an important role for the α subunit in inhibition. Such a role has already been suggested from experiments using S49 cyc$^-$ cells (Hildebrandt et al., 1984b).

The inhibition of the N_s(GppNHp)-stimulatable C activity, by N_i, appears to extend into the steady-state phase of time courses of activity, with the extent of inhibition being strictly dependent on the $MgCl_2$ concentration in the assay incubation. Specifically, the inhibition falls off as the divalent metal ion concentration is increased. One simple explanation for this effect is that at increasing levels of $MgCl_2$ the following equilibrium is pushed further to the right so that at constant [N_i] maximal inhibition is observed at low concentrations of $MgCl_2$.

$$N_s \text{ inactive} \xrightleftharpoons{Mg^{2+},\ \text{guanine nucleotide}} N_s^* \text{ active}$$

This would be especially true if N_i is preferentially activated (with respect to N_s) at low levels of $MgCl_2$ as has been suggested in earlier studies (Hildebrandt et al., 1983; Cerione et al., 1985).

The observed Mg^{2+} concentration dependence of the N_i inhibition in this reconstituted system is consistent either with a $\beta\gamma$-deactivation model or with a model where N_i, by directly interacting with C, reduces the affinity of N_s^* for C. In the $\beta\gamma$-deactivation model, a prerequisite assumption is that the $N_s \rightarrow N_s^*$ transition reflects subunit dissociation (into the α_s and $\beta\gamma$ species). Increased levels of $\beta\gamma$ from the activated N_i would then inhibit $N_s^*(\alpha_s)$ stimulation of C activity by pushing the $N_s \rightleftharpoons N_s^*$ equilibrium to the left. However, increasing Mg^{2+} concentrations (in the presence of GppNHp) would in turn increase [N_s^*] and thus serve to overcome the effect of $\beta\gamma$. Similarly, increasing [Mg^{2+}] could overcome an inhibition by N_i if the inhibition were due to an interaction between N_i and C which then reduced the affinity of N_s^* for C. In this case, sufficiently increasing [N_s^*] (by increasing [Mg^{2+}]) could allow N_s^*–C formation to occur even though the affinity for this interaction has been reduced. The ability of N_i to directly interact with C would also be consistent with the results observed in the cyc$^-$ mutants if, in these cases, an N_i–C interaction in some manner modulates the *intrinsic* activity of the cyc$^-$ catalytic (C) moiety. Further studies in which the effects of the pure holoprotein are compared to effects obtained using their separated subunits should allow us to ultimately determine the relative role of these mechanisms in the inhibition of adenylate cyclase.

Finally, very recently, by inserting N_i together with pure β-adrenergic receptors, N_s, and C in phospholipid vesicles, we have been able to document a guanine nucleotide induced inhibition of both basal and agonist (isoproterenol) promoted adenylate cyclase activity (unpublished results). Since the inhibition of basal activity is much greater than that of isoproterenol-induced activity, the overall "fold" stimulation by the stimulatory agonist is markedly increased. These results raise interesting possibilities about the role of N_i not only in hormonal inhibition but also in hormonal stimulation of adenylate cyclase and further underscore the utility of such reconstituted systems in probing the underlying mechanisms of regulation of this complex system.

ACKNOWLEDGMENTS

We express our gratitude to Lynn Tilley for her assistance in the preparation of the manuscript.

REFERENCES

Asano, T., & Ross, E. M. (1984) *Biochemistry 23*, 5468–5471.
Asano, T., Pedersen, S. E., Scott, C. W., & Ross, E. M. (1984) *Biochemistry 23*, 5460–5467.
Baehr, W., Morita, E. A., Swanson, R. J., & Applebury, M. L. (1982) *J. Biol. Chem. 257*, 6452–6460.
Bitensky, M. W., Wheeler, M. A., Rasenick, M. M., Yamazaki, A. Stein, P. J., Halliday, K. R., & Wheeler, G. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3408–3412.
Bokoch, G. M., Katada, T., Northup, J. K., Hewlett, E. L., & Gilman, A. G. (1983) *J. Biol. Chem. 258*, 2072–2075.
Cerione, R. A., Sibley, D. R., Codina, J., Benovic, J. L. Winslow, J., Neer, E. J. Birnbaumer, L., Caron, M. G., & Lefkowitz, R. J. (1984) *J. Biol. Chem. 259*, 9979–9982.
Cerione, R. A., Staniszewski, C., Benovic, J. L., Lefkowitz, R. J., Caron, M. G., Gierschik, P., Somers, R., Spiegel, A. M., Codina, J., & Birnbaumer, L. (1985) *J. Biol. Chem. 260*, 1493–1500.
Codina, J., Hildebrandt, J. D., Iyengar, R., Birnbaumer, L., Sekura, R. D., & Manclark, C. R. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4276–4280.
Codina, J., Hildebrandt, J. D., Sekura, R. D., Birnbaumer, M., Bryan, J., Manclark, R., Iyengar, R., & Birnbaumer, L. (1984) *J. Biol. Chem. 259*, 5871–5886.
Gilman, A. G. (1984) *Cell (Cambridge, Mass.) 36*, 577–579.
Hanski, E., & Gilman, A. G. (1982) *J. Cyclic Nucleotide Res. 8*, 323–336.
Hanski, E., Sternweis, P. C., Northup, J. K., Dromerick, A. W., & Gilman, A. G. (1981) *J. Biol. Chem. 256*, 12911–12919.
Hildebrandt, J. D., Hanoune, J., & Birnbaumer, L. (1982) *J. Biol. Chem. 257*, 14723–14725.
Hildebrandt, J. D., Codina, J., Risinger, R., & Birnbaumer, L. (1983) *J. Biol. Chem. 258*, 13141–13147.
Hildebrandt, J. D., Codina, J., Risinger, R., & Birnbaumer, L. (1984a) *J. Biol. Chem. 259*, 2039–2042.
Hildebrandt, J. D., Codina, J., & Birnbaumer, L. (1984b) *J. Biol. Chem. 259*, 13178–13185.
Iyengar, R., Abramowitz, J., Bordelon-Riser, M. E., & Birnbaumer, L. (1980) *J. Biol. Chem. 255*, 3558–3564.
Kanaho, Y., Tsai, S.-C., Adamik, R., Hewlett, E. L., Moss, J., & Vaughan, M. (1984) *J. Biol. Chem. 259*, 7378–7381.
Katada, T., Bokoch, G. M., Smigel, M., Ui, M., & Gilman, A. G. (1984) *J. Biol. Chem. 259*, 3586–3595.
Litman, B. J. (1982) *Methods Enzymol. 81*, 150–153.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Northup, J. K., Sternweis, P. C., Smigel, M. D., Schleifer, L. S., Ross, E. M., & Gilman, A. G. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6516–6520.
Northup, J. K., Smigel, M. D., Sternweis, P. C., & Gilman, A. G. (1983) *J. Biol. Chem. 258*, 11369–11376.
Salomon, Y., Londos, C., & Rodbell, M. (1974) *Anal. Biochem. 58*, 541–548.
Sternweis, P. C., Northup, J. K., Smigel, M. D., & Gilman, A. G. (1981) *J. Biol. Chem. 256*, 11517–11526.
Strittmatter, S., & Neer, E. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6344–6348.
Wald, G., & Brown, P. K. (1973) *J. Gen. Physiol. 37*, 189–200.