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Topical Review

Reconstitution of the β-Adrenergic Receptor

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Introduction

Of the several second messenger generating systems which serve to transmit signals across the plasma membrane the best characterized is the hormone-receptor-adenylate cyclase system (Lefkowitz, Stadel & Caron, 1983). Such systems consist of three distinct types of components which are portrayed schematically in Fig. 1. These components are: (i) a specific hormone receptor which may either stimulate or inhibit the enzyme; (ii) two special classes of regulatory proteins termed guanine nucleotide regulatory proteins which bind GTP and thereby mediate the hormone-receptor regulation of adenylate cyclase activity, and (iii) the catalytic moiety of adenylate cyclase itself. The two types of guanine nucleotide regulatory proteins denoted as N_s and N_i refer to distinct proteins which appear to couple respectively the stimulatory and the inhibitory receptors to the enzyme (Gilman, 1984).

Many different types of receptors can affect the activity of the adenylate cyclase system. Several dozen stimulatory receptors and at least a dozen inhibitory receptors have been identified to date. Very little is known about the molecular characteristics of these receptors. Only the β-adrenergic receptor for catecholamines has been purified and characterized to any extent. It is an integral membrane glycoprotein of $M_r \sim 64{,}000$ daltons as isolated from mammalian tissues (Lefkowitz et al., 1983). In contrast, the receptor from turkey erythrocytes appears to be of smaller size with two major peptides visualized by SDS-polyacrylamide gel electrophoresis. These peptides are of about 40,000 and 50,000 daltons (Shorr et al., 1982). Such isolated receptor peptides can be shown to bind β-adrenergic ligands with all the appropriate specificity and stereospecificity characteristics which would be expected from the well-defined pharmacology of these receptors.

The guanine nucleotide regulatory proteins, which have been shown to couple receptors such as the β-adrenergic receptor to the catalytic moiety of adenylate cyclase, have also been purified and characterized. The stimulatory nucleotide regulatory protein of the adenylate cyclase system, N_s, is heterotrimeric with an α subunit of $M_r \cong 43{,}000$, β of $M_r \cong 35{,}000$, and γ of $M_r \cong 5{,}000$–$10{,}000$ (Sternweis et al., 1981; Codina et al., 1984). The structure of N_i is similar except that the α subunit is only 41,000 daltons (Bokoch et al., 1984; Codina et al., 1984). For both proteins the α subunit contains the GTP binding site as well as the GTPase activity that hydrolyzes GTP to GDP. The α subunit is, in each case, a substrate for ADP ribosylation by specific toxins; cholera toxin in the case of N_s and *Bordetella pertussis* toxin in the case of N_i. These proteins appear to be members of a larger family of guanine nucleotide regulatory proteins which may be involved in coupling all sorts of receptors to a wide array of physiological effector systems (Gilman, 1984). A third member of this family, which has been purified and well characterized, is transducin, the guanine nucleotide regulatory protein of the vertebrate visual transduction system.

The structure of the catalytic component of the adenylate cyclase system has not been extensively characterized since it has only recently yielded to purification efforts. Recent reports suggest it is a membrane glycoprotein of ~150,000 daltons (Pfeuffer et al., 1985). Partially purified preparations of the catalyst, which are nonetheless resolved from the other components of the system, can be readily prepared by gel filtration chromatography (Ross et al., 1978; Strittmatter & Neer, 1980).

The availability of purified preparations of the

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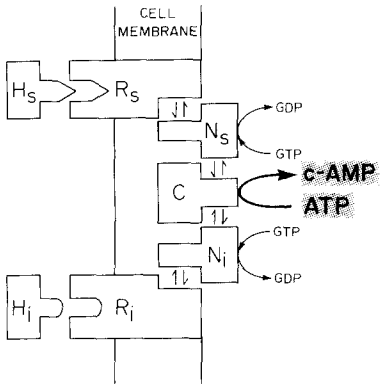

Fig. 1. Schematic representation of a hormone-responsive adenylate cyclase system. *H* = hormone; *R* = receptor; *N* = guanine nucleotide regulatory component; *C* = catalytic unit; *s* = stimulatory; *i* = inhibitory

different components of the catecholamine-responsive adenylate cyclase system has opened the way for direct studies of the functional interactions occurring between these components. A critical step in performing such experiments is the development of reconstitution approaches whereby the detergents are removed from each of the solubilized and purified protein components and then the isolated proteins are reinserted into a lipid milieu (phospholipid vesicle). Such methods have been developed only over the past several years (Pedersen & Ross, 1982; Cerione et al., 1983*a*,*b*, 1984*a*; Brandt et al., 1983). They have already provided great insights into the mechanisms by which hormones affect adenylate cyclase activity through receptor-mediated mechanisms as well as into the processes that regulate the function of this system. This article will review our recent work in developing and exploiting systems for reconstitution of β-adrenergic receptors.

Insertion of β-Adrenergic Receptors into Phospholipid Vesicles

The successful functional insertion of a purified protein into a lipid milieu involves, in effect, the transfer of the protein from the solubilizing detergent (micelle) into phospholipid vesicles. In the case of the β-adrenergic receptor this is particularly difficult since digitonin is the only detergent that solubilizes this receptor in a form that still binds ligands and which thus permits significant purification by affinity chromatography (Caron & Lefkowitz, 1976). The low critical micelle concentration of this detergent (0.1–0.3 mM) makes detergent removal difficult. Effective elimination of the digitonin is necessary both to permit proper vesicle formation and to allow the insertion of the receptor polypeptides as well as to allow coupling between the receptor and other components of the adenylate cyclase system. Various reconstitution approaches have been described which successfully eliminate detergent and effectively reconstitute β-adrenergic receptors in lipid vesicles. In general the inclusion of an additional detergent during the initial reconstitution incubation of the β-adrenergic receptors with preformed phospholipid vesicles markedly increases the efficiency of reconstitution (Pedersen & Ross, 1982; Cerione et al., 1983*a*,*b*, 1984*a*; Brandt et al., 1983). The best results have been obtained using octyl glucoside and sodium deoxycholate since both of these detergents are more effective at initially solubilizing the preformed vesicles as well as more easily removed compared to digitonin (thus improving the chances of reforming the vesicle structure around the receptor). The actual lipids used for reconstitution do not appear to be critical. Successful reconstitutions have been performed with dimyristoyl phosphatidylcholine, crude soybean phosphatidylcholine or crude lipid extracts derived from the membranes from which the receptors were originally prepared. The detergent is usually removed by Sephadex chromatography (Pedersen & Ross, 1982; Brandt et al., 1983) or by using various detergent removing resins such as SM-2 or Extracti-gel® (Cerione et al., 1983*a*,*b*, 1984*a*). In general, the efficiencies of reconstitution of partially purified as well as entirely pure receptor preparations are essentially identical, suggesting that the receptor binding protein purified to apparent homogeneity contains all the necessary components for insertion into phospholipid bilayers.

Functionality of Reconstituted β-Adrenergic Receptors

The major advantage of reconstituted receptor preparations is that their biological activity can be assessed in a functional context. It should be stressed that the availability of ligand binding approaches for measuring β-adrenergic receptor binding has provided an enormous amount of information about the binding function of the receptor (Stiles, Caron & Lefkowitz, 1984). However, receptors are essentially bifunctional proteins and in addition to binding ligands must also activate physiological response mechanisms. In the case of β-adrenergic and many other membrane-bound receptors this effector mechanism is the adenylate cyclase system. It is only through the use of reconsti-

tuted receptor systems that this second activating function of receptors can be assessed.

Over the past few years several different approaches have been developed for assessing the functionality of reconstituted β-adrenergic receptor polypeptides. The first approach was patterned after the early work of Schramm and coworkers where they fused whole cells containing β-adrenergic receptors, but in which the *N* and *C* components of the cyclase had been inactivated chemically, to other cells which contained *N* and *C* but lacked β-adrenergic receptors (Schramm, 1979; Eimerl et al., 1980; Korner, Gilon & Schramm, 1982). They were able to show that in the membranes derived from the fused hybrid cells catecholamine-responsive adenylate cyclase was present. This documented the feasibility of functionally recoupling β receptors with the adenylate cyclase system. Accordingly, our initial strategy was to see if β-adrenergic receptors reconstituted in lipid vesicles could be successfully recoupled to an adenylate cyclase system in a cell that lacked β-adrenergic receptors. For these experiments we chose the *Xenopus laevis* erythrocyte. These cells contain the adenylate cyclase enzyme and the stimulatory nucleotide binding regulatory protein but little or no β-adrenergic receptors. Thus, their adenylate cyclase activity is unresponsive to β-adrenergic agonists. The fusion technology was adapted from previously published methods for membrane cell fusion using polyethyleneglycol and lipids (Schramm, 1979; Eimerl et al., 1980; Korner et al., 1982; Strulovici, Stadel & Lefkowitz, 1983). As indicated in Fig. 2 we were able to demonstrate that the fusion of reconstituted pure hamster lung β-adrenergic receptors into the *Xenopus* erythrocyte established a significant stimulation of the hybrid cyclase activity by isoproterenol (Cerione et al., 1983*a*). It can also be noted that the stimulation was completely blocked by the β-adrenergic antagonist propranolol and was not observed when vesicles containing no receptor (receptor which was boiled) were added to the fusion system. Comparable amounts of β-adrenergic receptor binding activity obtained from preparations of widely varying degrees of purity and from several different tissues and species give comparable degrees of stimulation of adenylate cyclase activity in the hybrid cells (Cerione et al., 1983*a*,*b*). These results indicate that the ability of the receptor to couple to adenylate cyclase is not altered during either the purification or the reconstitution procedures. The fact that similar responses were obtained with receptors isolated from tissues as diverse as frog erythrocytes, hamster lungs, and guinea pig lungs suggests that the coupling sites on these receptor polypeptides are highly conserved.

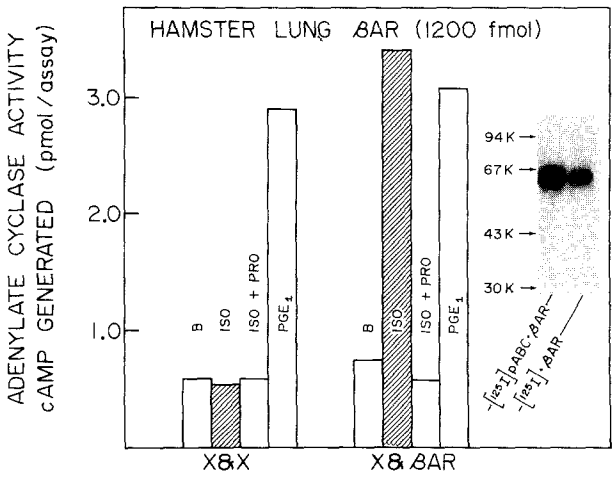

Fig. 2. Adenylate cyclase activity in fused hybrids of reconstituted pure hamster lung β-adrenergic receptor and *Xenopus laevis* erythrocytes. *B*, basal; *ISO*, isoproterenol (50 μM); *ISO + PRO*, isoproterenol (50 μM) + propranolol (50 μM); PGE_1, prostaglandin E_1 (3 μM). *X & X* represents *X. laevis* erythrocytes fused with phospholipid vesicles containing a mixture of soybean phosphatidylcholine and dimyristoyl phosphatidylcholine without β-adrenergic receptor. *X & βAR* represents pure hamster lung receptor inserted into the above phospholipid vesicles (1200 fmol) and fused with the *X. laevis* erythrocytes. Membranes of the resulting hybrids were prepared using freeze-thaw lysis. Adenylate cyclase assays were performed using 0.1 mM ATP as described (Cerione et al., 1983*a*). The assay data are reported as pmol cyclic AMP generated in a 30-min incubation period (30°C). *Inset:* Autoradiogram of SDS-PAGE of the photoaffinity labeled ([^{125}I]*p*-azidobenzylcarazolol) and iodinated purified β-adrenergic receptor preparation used in these experiments. Data was obtained from Cerione et al. (1983*a*)

Reconstitution of Functional Interactions between Pure β-Adrenergic Receptors and the Pure N_s in Phospholipid Vesicles

Our own group (Cerione et al., 1984*a*,*b*, 1985) and that of Ross (Brandt et al., 1983; Asano et al., 1984*a*,*b*; Asano & Ross, 1984) have succeeded in establishing conditions for studying the direct interactions of β-adrenergic receptors and purified N_s proteins in reconstituted phospholipid vesicles. Such studies required the development of procedures for the coinsertion of the pure proteins into phospholipid vesicles. We found that the most effective way of accomplishing this is to incubate these components together with soybean phosphatidylcholine and octyl glucoside and to then reduce the detergent concentration by chromatography on Extracti-gel® columns. This detergent-adsorbing matrix appears to be more effective for such experiments than the SM-2 beads. This is probably due to its more effective capacity for reducing detergent levels. It is essential that the levels of detergent

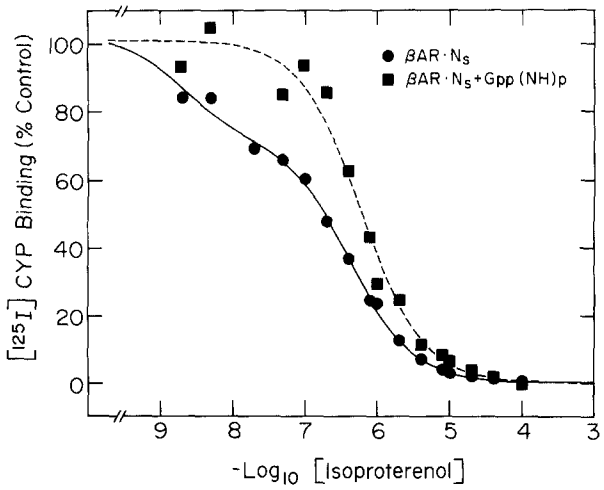

Fig. 3. (−)Isoproterenol competition for [^{125}I]iodocyanopindolol ([^{125}I]CYP) binding to phospholipid vesicles containing pure *βAR* and pure N_s. Guinea pig lung *βAR* (6.7 pmol) and human erythrocyte N_s (6.0 pmol) were incubated with phospholipids in reconstitution incubations as described in Cerione et al. (1984*a*), and then the isolated phospholipid vesicles were assayed in the absence (●) and presence (■) of 5′-guanylyl-imidodiphosphate for binding to the *βAR* as described (Cerione et al., 1984*a*). Each point represents the mean of duplicate determinations, and the data shown is representative of two experiments. Data was obtained from Cerione et al. (1984*a*)

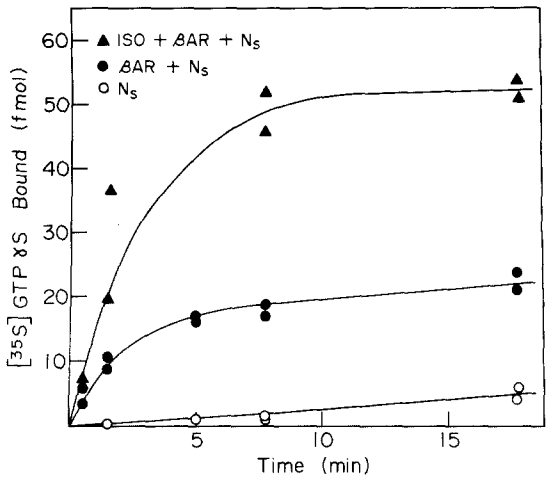

Fig. 4. [^{35}S]GTPγS binding to phospholipid vesicles containing pure *βAR* and pure N_s. Human erythrocyte N_s (15.3 pmol) was added to reconstitution incubations alone or together with guinea pig lung *βAR* (10.1 pmol) as described in Cerione et al. (1984*a*). Binding experiments were performed on 10 μl aliquots of phospholipid vesicles containing N_s alone (○), and N_s together with guinea pig lung *βAR* in the presence (▲) and absence (●) of 10^{-5} M (−)isoproterenol. The data shown is representative of three experiments. Data was obtained from Cerione et al. (1984*a*)

present in the receptor and N_s preparations be very much reduced in such studies since the coupling of receptor and N_s is highly sensitive to detergent.

The functional interactions that occur between pure β-adrenergic receptors and pure N_s in such phospholipid vesicles can be monitored via several different types of activities. These activities are: (a) high affinity binding of β agonists to the receptor; (b) high affinity guanine nucleotide binding to N_s; and (c) GTPase activity in N_s.

Agonist Binding to Phospholipid Vesicles Containing β-Adrenergic Receptors and N_s

The characteristics of agonist binding to β-adrenergic receptors in intact membranes have been very well studied. The essential feature of such binding is that it is complex with the agonist binding to two seemingly discrete classes of receptor binding sites which usually differ in their affinities for agonist by about 100-fold. Moreover, when guanine nucleotides such as GTP or GppNHp are added to such incubations all the high affinity receptor binding sites appear to be converted to low affinity sites. This has the effect of shifting the agonist competition curve to the right and steepening it so that its slope factor or pseudo-Hill coefficient is now about 1 (Kent, DeLean & Lefkowitz, 1980). A series of biochemical investigations, which will not be reviewed here, have shown that low affinity binding sites consist of a binary complex of agonist and receptor, whereas the high affinity site consists of a ternary complex of agonist, receptor, and guanine nucleotide regulatory protein (Lefkowitz et al., 1983).

When agonist competition curves for the binding of the radiolabeled antagonist [^{125}I]iodocyanopindolol in phospholipid vesicles containing the β-adrenergic receptor and N_s were constructed, they were found to be complex and indicative of two classes of binding sites (Fig. 3). As in membranes, guanine nucleotides cause such curves to steepen and shift to the right, reflecting a reduction in the proportion of receptors in the agonist high affinity state with a corresponding increase in the percentage of receptors in the agonist low affinity state. For the data shown in Fig. 3, initially ~30% of the receptors were in the agonist high affinity state with a K_d of about 2 nM, and 70% were in the agonist low affinity state with a K_d of about 300 nM (Cerione et al., 1984*a*). In addition, the nonhydrolyzable analog GppNHp results in 100% of the receptors existing in the low affinity state. When the β receptor was inserted into phospholipid vesicles alone (that is, in the absence of N_s) the isoproterenol competition

curves were observed to be monophasic and already shifted to the right (*data not shown*) (Cerione et al., 1984*a*). These data indicate that the interactions of the pure β-adrenergic receptor and the pure stimulatory guanine nucleotide regulatory protein are sufficient to induce an agonist high affinity, guanine nucleotide-sensitive state of the receptor. The fact that only ~30% of the receptors form the high affinity receptor state in this reconstituted preparation as compared with usually about 60–70% in native membranes is presumably due to problems of access of receptors to the *N* proteins in the phospholipid vesicles (i.e., under these experimental conditions where the ratio of proteins to vesicles is <1).

[^{35}S]GTPγS Binding to Phospholipid Vesicles Containing the β-Adrenergic Receptor and N_s

Figure 4 indicates another approach to documenting functional interactions of β-adrenergic receptors and N_s in phospholipid vesicles. In these experiments the binding of [^{35}S]GTPγS, a nonhydrolyzable GTP analog, to the N_s protein was monitored. Under the experimental conditions used here, binding of the nucleotide to phospholipid vesicles containing N_s alone was very low. However, inclusion of the β-adrenergic receptor in these vesicles promoted as much as a fourfold increase in the extent of [^{35}S]GTPγS binding to N_s and the addition of isoproterenol increased the extent of binding an additional twofold. Moreover, addition of isoproterenol to such vesicles leads to an approximate fivefold increase in the rate of [^{35}S]GTPγS binding (Cerione et al., 1985).

GTPase Activity of Phospholipid Vesicles Containing the β-Adrenergic Receptor and N_s

The third way in which the functional interactions between the β-adrenergic receptors and N_s can be monitored in reconstituted phospholipid vesicles is by measuring the GTPase activity of N_s. As shown in Fig. 5, N_s alone in phospholipid vesicles is capable of only low levels of GTPase activity. However, the coinsertion of pure β-adrenergic receptors with the pure N_s increases this activity, with the extent of the increase being dependent on the amount of β receptor and N_s inserted into the phospholipid vesicles. In general, we have constructed vesicles that contain three- to fivefold excess of N_s over β receptors. In such vesicles the β-adrenergic agonist isoproterenol induces as much as a six- to 10-fold stimulation of the GTPase activity relative to that ob-

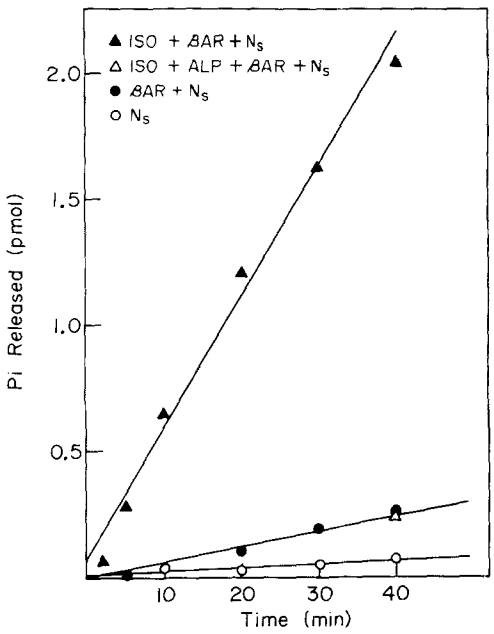

Fig. 5. GTPase activity of phospholipid vesicles containing pure βAR and pure N_s. Reconstitution incubations were performed as described in Fig. 4. GTPase activity was determined on 20 μl aliquots of the resuspended vesicles as described in Cerione et al. (1984*a*). ○, vesicles containing N_s alone; ●, vesicles containing N_s together with βAR; ▲, vesicles containing N_s together with βAR assayed in the presence of 10^{-5} M (−)isoproterenol, △, vesicles containing N_s together with βAR assayed in the presence of 10^{-5} M (−)isoproterenol plus 10^{-4} M (−)alprenolol. The data shown is representative of three experiments, and each point is the average of duplicate determinations. Data was obtained from Cerione et al. (1984*a*)

tained with receptor and N_s alone (Cerione et al., 1984*a*, 1985). The isoproterenol stimulation can be completely abolished by β antagonists such as alprenolol in a stereoselective manner. Moreover, agonists can stimulate the GTPase activity in this reconstituted system with the order of potency expected for the pure guinea pig lung receptor that belongs to the β_2 subclass of β-adrenergic receptors (Cerione et al., 1984*a*).

Specificity of β-Adrenergic Receptor and Guanine Nucleotide Regulatory Binding Protein Interactions

As noted earlier, N_s is only one member of a larger family of guanine nucleotide regulatory proteins (Gilman, 1984). All members of this family may not yet have been discovered. Among those which are known in addition to N_s are N_i, the inhibitory guanine nucleotide regulatory protein, involved in inhibition of adenylate cyclase, N_o, a very abundant

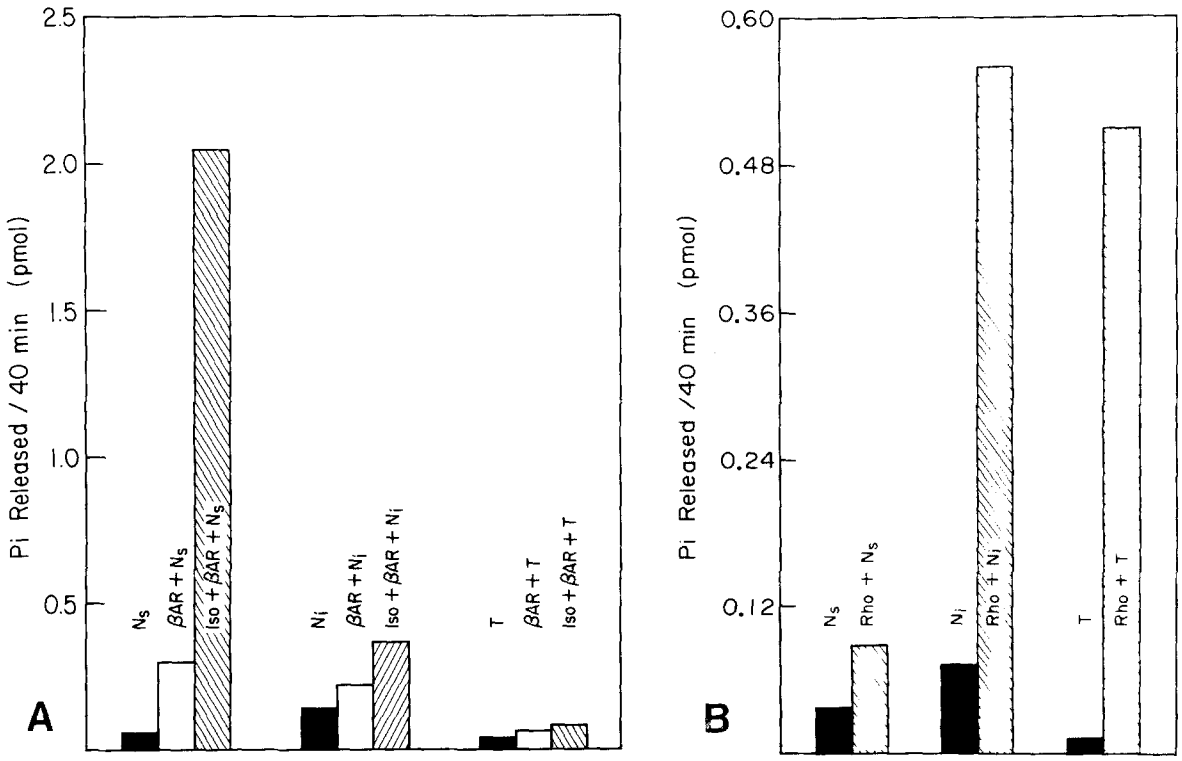

Fig. 6. (*A*): GTPase activity in phospholipid vesicles containing pure preparations of βAR and N_s, N_i or transducin (T). Reconstitution incubations containing βAR and N_s were performed as described in Figs. 4 and 5. The βAR, N_i and βAR, T incubations contained 10.5 pmol human erythrocyte N_i and 0.8 μg (≅8–10 pmol) transducin, respectively. GTPase experiments were performed as described in Cerione et al. (1984*a*). Each bar represents the results of several experiments. (−)Isoproterenol = 10^{-5} M. (*B*): GTPase activity in phospholipid vesicles containing pure preparations of rhodopsin (*Rho*) and N_s, rhodopsin and N_i, or rhodopsin and transducin (T). Reconstitution incubations were initiated with 36 pmol rhodopsin, 12.2 pmol N_s, 10.5 pmol N_i and 0.8 μg transducin (≅8–10 pmol). GTPase assays were performed as described in Cerione et al. (1985) on 20 μl aliquots of vesicles containing the different proteins as indicated, for 40 min at 30°C. Each bar graph represents the average of duplicate experiments, and the data shown is representative of two experiments. Data was obtained from Cerione et al. (1985)

guanine nucleotide regulatory protein found in brain, which is of unknown physiological function (Neer, Lok & Wolf, 1984; Sternweis & Robishaw, 1984), and transducin, the protein that couples rhodopsin to cyclic GMP phosphodiesterase as part of the visual transduction process in rod outer segments (Gilman, 1984; Stryer, Hurley & Fung, 1981).

In order to obtain information pertinent to the general mechanism of receptor-nucleotide binding regulatory protein coupling, as well as information concerning the specificity of these interactions, we also examined several other of these proteins for their ability to interact with the β-adrenergic receptors in reconstituted systems (Cerione et al., 1985). The results of these studies are shown in Fig. 6*A* and *B*. It can be seen from Fig. 6*A* that although the β-adrenergic receptor is able to interact with N_i, as documented by an agonist-promoted increase in GTPase in this system, the extent of this interaction is much less than that of N_s with its homologous receptor, i.e., the β-adrenergic receptor. For transducin there is almost no interaction. By contrast, as shown in Fig. 6*B* the retinal light receptor rhodopsin is able to stimulate the GTPase activity of N_i and transducin essentially equally well, while it barely stimulates that of N_s. However, rhodopsin actually induces a 40-fold stimulation of GTPase activity in transducin compared to about an eightfold stimulation of the activity in N_i. This likely reflects the increased capability of N_i alone to bind and hydrolyze GTP at the concentration of magnesium (10 mM) used in these experiments as compared to transducin alone (Cerione et al., 1985).

The Table lists the turnover numbers for GTPase activity for N_s, N_i and transducin in phosphatidylcholine vesicles after reconstitution alone, or together with either the β-adrenergic receptor or rhodopsin. These turnover numbers represent the total amount of P_i released (during a 40-min assay) divided by the amount of N protein-guanine nucleotide complex formed during the 40-min incubation (as determined by [^{35}S]GTPγS binding). These turn-

Table. Comparison of GTPase turnover numbers for the different nucleotide regulatory proteins in the presence and absence of receptors[a]

Component	GTPase turnover[b] (mol P_i released min − mol N)	n
1. N_s	0.07 ± 0.04	3
2. N_i	0.10 ± 0.02	5
3. Transducin	0.06 ± 0.02	3
4. N_s + βAR	0.13 ± 0.02	10
5. N_s + βAR + Iso	0.71 ± 0.11	10
6. N_i + βAR	0.10 ± 0.02	6
7. N_i + βAR + Iso	0.12 ± 0.02	8
8. Transducin + βAR	0.06 ± 0.01	5
9. Transducin + βAR + Iso	0.07 ± 0.02	4
10. Transducin + Rhodopsin[c]	0.14 − 0.22	2
11. Transducin + Rhodopsin[d]	0.30 − 0.71	2
12. N_i + Rhodopsin[e]	0.25 − 0.36	2
13. N_i + Rhodopsin[d]	0.64	1
14. N_s + Rhodopsin[e]	0.12 − 0.15	2

[a] [Data from Cerione et al. (1985).]

[b] The values for the turnover numbers listed in column two were determined from the results of GTPase experiments and [^{35}S]GTPγS binding studies performed at 30°C (for 40 min) as described in Cerione et al. (1985). In these experiments the concentrations of the following agents were: [γ^{32}P] GTP = 100–200 nM; [^{35}S]GTPγS = 100 nM, $MgCl_2$ = 10 mM. The quantities of nucleotide regulatory proteins (N) were determined by [^{35}S]GTPγS binding.

[c] The rhodopsin concentration in these experiments was 18–36 pmol/ml vesicles.

[d] The rhodopsin concentration in these experiments were 135 pmol/ml vesicles.

[e] The rhodopsin concentrations in the two experiments shown were 18 and 135 pmol/ml.

n = number of experiments.

In all cases, the values listed are the means (± SE) of the results obtained from the different experiments.

over numbers thus give a relative measure of the catalytic efficiency of the nucleotide binding regulatory protein in the presence and absence of receptors. Several interesting insights emerge from these data. First, the average catalytic turnovers for all three nucleotide regulatory proteins in the absence of receptors are similar. The β-adrenergic receptor alone has essentially no effect on the catalytic turnover of either N_i or of transducin and only a small effect on the turnover of N_s. However, the addition of isoproterenol causes a marked increase in the catalytic turnover of N_s while causing a much smaller effect on the turnovers of N_i and transducin. Taken together these results indicate that the preferred order of N protein interactions with the β-adrenergic receptor is $N_s \gg N_i >$ transducin. Of further interest is the fact that the upper range of the rhodopsin-stimulated GTPase activity of N_i and transducin is very similar to the turnover number obtained for isoproterenol-stimulated GTPase activity of N_s. This suggests that all of these N proteins possess a common upper limit of catalytic efficiency.

Reconstitution of a Hormone-Sensitive Adenylate Cyclase System

The ultimate aim of reconstitution studies of hormone-sensitive adenylate cyclase is to establish a completely reconstituted hormone-responsive adenylate cyclase from its isolated components. We have recently been able to establish such a system in which both the β-adrenergic receptor and N_s proteins are pure and the cyclase preparation (C), though not pure, is resolved by gel filtration chromatography from receptors and guanine nucleotide regulatory proteins (Cerione et al., 1984*b*). All of the components are co-reconstituted into phosphatidylcholine vesicles using procedures similar to those developed for the reconstitution of the receptors and N_s. When the resolved catalytic unit is inserted into phospholipid vesicles alone a forskolin-stimulatable cyclase activity is observed. However, this activity is not further stimulated by guanine nucleotides or sodium fluoride. When the C preparations are co-inserted with pure human erythrocyte N_s into the phospholipid vesicles, the nonhydrolyzable GTP analog GppNHp leads to a fourfold stimulation of enzyme activity. Under these conditions there is little or no stimulation of the activity with GTP and no additional stimulation by guanine nucleotides upon addition of isoproterenol to the assay incubations (Cerione et al., 1984*b*).

As shown in Fig. 7 this adenylate cyclase system becomes hormone-responsive when pure N_s and resolved C are inserted into phosphatidylcholine vesicles together with pure β-adrenergic receptors. The reconstituted system appears to show all the appropriate features of the *in vivo* system. The stimulation of the reconstituted adenylate cyclase activity by isoproterenol can be completely blocked by β antagonists in a stereoselective manner. Agonist stimulation is inversely dependent on magnesium concentration in the assay as earlier reported for glucagon stimulation of adenylate cyclase activity in intact membranes. Thus, at higher magnesium concentrations enzyme activity rises and becomes less responsive to hormonal stimulation. These results appear to reflect, at increasing magnesium concentrations, the direct stimulation of C by the divalent metal and the stimulation of C by $N_s \cdot$ GTP complexes which can form to an increasing extent

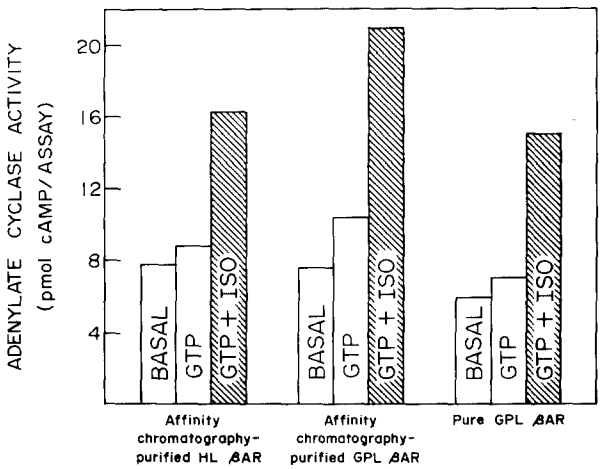

Fig. 7. Adenylate cyclase activity in phospholipid vesicles containing bovine caudate *C*, pure human erythrocyte N_s, and purified *βAR* preparations. *HL*, hamster lung; *GPL*, guinea pig lung. Resolved *C* preparation, 12–29 pmol of N_s, and 10–22 pmol of *βAR* were reconstituted as described in Cerione et al. (1984*b*), and the phospholipid vesicles (20 μl) were assayed for cyclic AMP production (50 μl) at a final $MgCl_2$ concentrations of 2 mM (when using pure guinea pig lung *βAR* or 5 mM when using partially purified *βAR*). In all cases (−)isoproterenol = 10^{-4} M. GTP = 10^{-5} M when affinity chromatography purified hamster lung *βAR* was used; GTP = 10^{-4} M in all other cases. Maximal stimulations of adenylate cyclase activity are obtained at 10^{-5} M GTP. Each set of data shown represents the mean of duplicate determinations. Data was obtained from Cerione et al. (1984*b*)

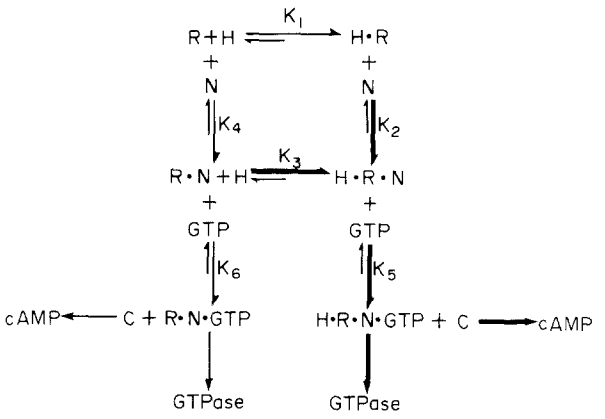

Fig. 8. β-Adrenergic receptor-coupled adenylate cyclase system. *R* depicts the β-adrenergic receptor, *N* is the stimulatory nucleotide-binding regulatory protein, *C* is the catalytic moiety of adenylate cyclase, and *H* is a hormone agonist. K_1–K_4 are the equilibrium constants for the different hormone-receptor or receptor-*N* interactions, while K_5 and K_6 are the equilibrium constants for both GTP binding to *RN* and *HRN* complexes and the subsequent changes in *N* that result in activation of adenylate cyclase or in GTPase activity

even in the absence of receptor and N_s interactions (Cerione et al., 1984*b*). This effect suggests that isoproterenol stimulation of adenylate cyclase activity (as well as stimulation of GTP binding to, and GTPase activity in, N_s) may reflect an increase in the affinity of N_s for magnesium.

The reconstitution studies described above have broad significance at at least two levels. First, they provide an approach to assaying the functionality of an isolated biological receptor. Below we will provide an example of how such assays can be used to dissect the mechanisms of physiological regulation of receptor function as, for example, after desensitization. Secondly, and equally important, such systems have already begun to provide us with significant information regarding the molecular basis of hormone activation of the enzyme. The results obtained thus far are consistent with the general scheme shown in Fig. 8. For simplicity, the potential dissociation of N_s into its component subunits is not depicted nor is any direct binding of GTP to N_s in the absence of receptor and N_s interactions. We have also excluded details regarding the direct interactions between N_s and *C*.

As described above, studies using the two-component reconstitution system have demonstrated that the interactions of the pure β-adrenergic receptor with pure N_s are sufficient to induce a high affinity state for agonists in *R*. Thus, K_3 is greater than K_1. Moreover, the results from both GTPase experiments and [^{35}S]GTPγS binding studies indicate that *R* and *N* can interact even in the absence of agonist. Therefore, the upper box of the scheme can be completed with the equilibrium described by K_4. Microscopic reversibility requires that K_2 be greater than K_4, i.e., *N* binds much more tightly to HR complexes than to R alone. Thus, the GTP binding interaction leading to activation, as described by K_5, is favored relative to that described by K_6, resulting in the observed agonist-promoted increases in [^{35}S]GTPγS binding, GTPase activity and adenylate cyclase activity. The rate constant for agonist promoted binding of [^{35}S]GTPγS to N_s (about 1 min^{-1}) is similar to the turnover number for agonist stimulated GTPase activity (about 0.7 min^{-1}) under identical experimental conditions. This is consistent with the suggestion that GTP binding to N_s may be the rate limiting step for GTPase activity. The actual mechanism by which the agonist-receptor complex promotes guanine nucleotide binding to N_s may involve a conformational change that increases the affinity of N_s for magnesium, which is a necessary cofactor for both GTP binding and GTPase activity.

The physiological significance, if any, of the weak interaction of β-adrenergic receptors with N_i (Cerione et al., 1985; Asano et al., 1984*a*) remains to be determined. Such crossreactivity might be-

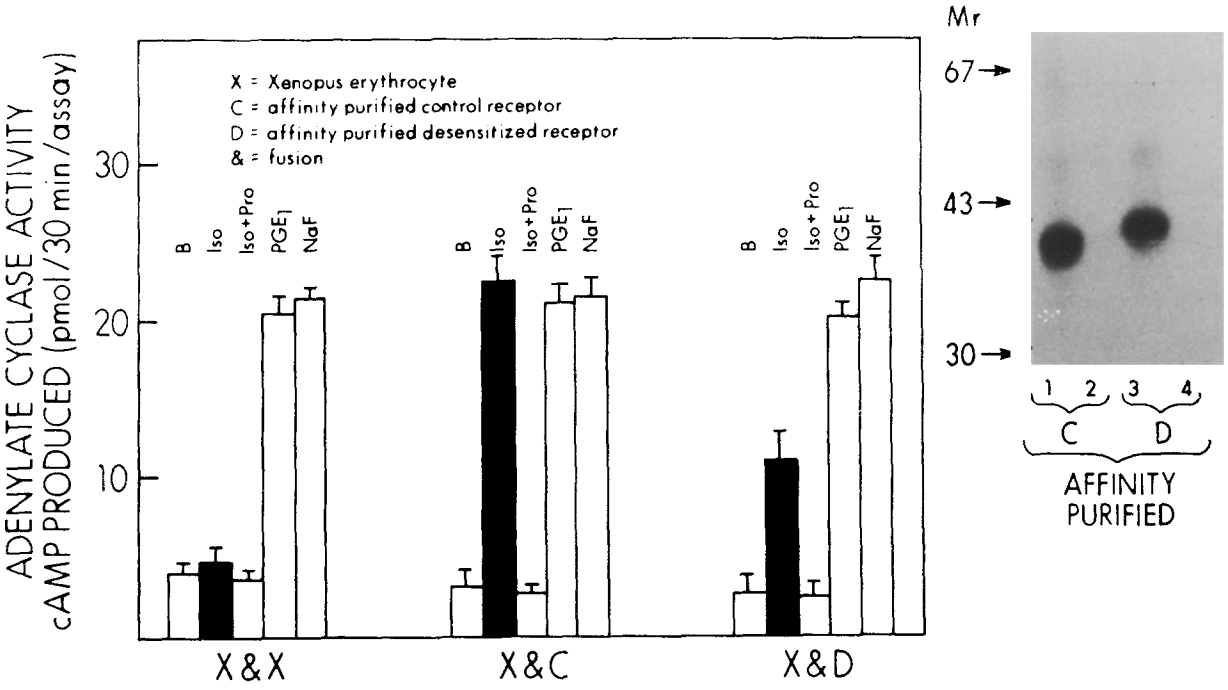

Fig. 9. Fusion of *Xenopus laevis* erythrocytes with affinity chromatography-purified and reconstituted β-adrenergic receptors from control and densensitized turkey erythrocytes. *B*, basal; *ISO*, isoproterenol (50 μM); *ISO + PRO*, isoproterenol (50 μM) + propranolol (50 μM); PGE_1, prostaglandin E_1 (3 μM); NaF, sodium fluoride (100 mM). The insertion of turkey erythrocyte β-adrenergic receptor into phospholipid vesicles and the fusion of these vesicles with *X. laevis* erythrocytes was as described (Strulovici et al., 1984). The same amount of inserted receptor (control and desensitized) (~1 pmol) were fused into the acceptor cells in these experiments. *X & X* represents *X. laevis* erythrocytes which were put through the fusion procedure in the absence of lipid vesicles. *X & C* represents the fusion of turkey erythrocyte *βAR*, isolated from control membranes, with *X. laevis* erythrocytes; *X & D* represents the fusion of turkey erythrocyte *βAR*, isolated from desensitized membranes, with *X. laevis* erythrocytes. Data was obtained from Strulovici et al. (1984)

come significant *in vivo* since N_i appears to be a much more abundant protein than is N_s (Gilman, 1984). This potential crossreactivity will have to be kept in mind in future mechanistic studies. The results with transducin strengthen the notion that transducin and N_i are functionally much more equivalent than are either of these proteins and N_s.

Reconstitution Studies and Desensitization of the β-Adrenergic Receptor

The ability to assay the function of isolated receptors in reconstituted systems opens a whole new approach to the assessment of the molecular mechanisms underlying physiological regulation of receptor function. Just as the advent of ligand binding studies 10–15 years ago opened the door to the assessment of the binding function of receptors under a wide variety of conditions, so the development of reconstitution approaches now permits assessing the activating function of these receptors under comparable circumstances. As an example of this approach we will present some recent data concerning the functionality of isolated receptors that have been desensitized by prior exposure to β-adrenergic agonists.

We will not review here the extensive literature (reviewed in Harden, 1983; Stiles et al., 1984; Sibley & Lefkowitz, 1985) dealing with the subject of desensitization of β-adrenergic and other receptors. Suffice it to say that when hormone-responsive adenylate cyclase systems are chronically stimulated by agonists, hormones, or drugs, they demonstrate a markedly reduced capacity to respond to subsequent challenge with that agonist. This phenomenon is referred to as desensitization. Several different types of desensitization have been delineated. In one form, termed "homologous," the loss of hormone responsiveness is quite specific and involves alterations only in subsequent challenge by the same agonist as was used for the desensitization. All other hormonal effectors acting through distinct receptors display normal responsiveness. This form of desensitization appears not to be cyclic-AMP mediated. In contrast, in "heterologous" desensitization stimulation of the cell by any agonist leads to loss of responsiveness to subse-

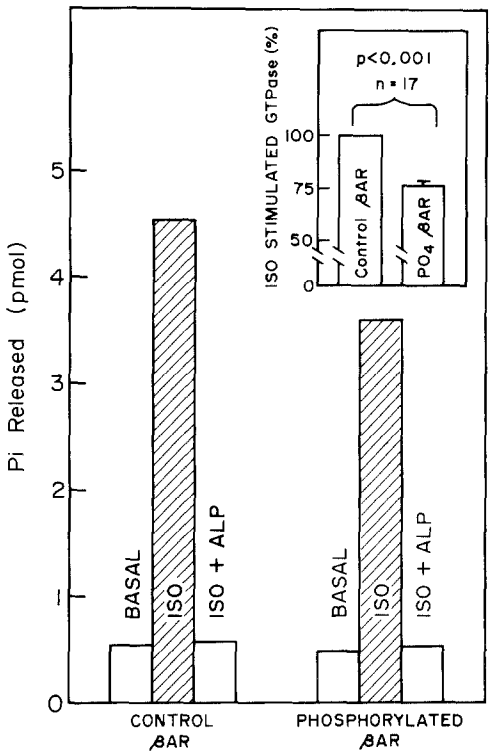

Fig. 10. GTPase activity in phospholipid vesicles containing N_s and phosphorylated or control receptor. The β-adrenergic receptor (5–30 pmol), N_s (3–25 pmol) and protein kinase catalytic unit (100–250 pmol) were added to reconstitution incubations as described (Benovic et al., 1985). Aliquots of the inserted receptor were then either phosphorylated with ATP, as outlined previously (Benovic et al., 1985), or kept as control samples, and then assayed for GTPase activity. The result shown is representative of 17 experiments and was taken from Benovic et al. (1985). *Inset:* Isoproterenol-stimulated GTPase activity was determined by subtracting the basal activity from the activity measured in the presence of 20 μM (−)isoproterenol. Data were normalized to give 100% activity for control receptor. The GTPase activities for phosphorylated receptor (PO_4 *βAR*) were 76.3 ± 1.3% (mean ± SE, $P < 0.001$, $n = 17$) of control

quent stimulation by several different classes of agonists working through distinct receptors. This form of desensitization appears to be mediated at least in part by cyclic AMP. Whereas in homologous desensitization the β-adrenergic receptors appear to be functionally sequestered away from both the cell surface and their physiological effector, the $N_s \cdot C$ system, in heterologous desensitization receptors appear to remain in the plasma membrane and become functionally uncoupled.

Recently several lines of evidence have emerged which strongly support the notion that in heterologous desensitization the functional uncoupling of the receptors may be associated with their phosphorylation, both by the cyclic AMP-dependent protein kinase and possibly by other protein kinases as well (reviewed in Benovic & Lekfowitz, 1985). Most of the reported work has been done with simple avian erythrocyte systems. When such cells are exposed to β agonists there is a 50–60% reduction in catecholamine-stimulated adenylate cyclase as well as lesser reductions in other parameters of enzyme activity (Hoffman, Mullikin-Kilpatrick & Lefkowitz, 1979; Simpson & Pfeuffer, 1980). This cell appears to represent a model of heterologous desensitization. A similar though less pronounced form of this desensitization can be evoked simply by incubating the cells with cyclic AMP analogs (Stadel et al., 1981). The desensitization is associated with about a threefold increase in the phosphate content of the receptors (Stadel et al., 1983; Sibley et al., 1983) such that after maximum desensitization there are 2–3 mol phosphate/mol receptor.

In order to assess the functionality of the desensitized and phosphorylated receptors, we isolated them from desensitized turkey erythrocytes and compared their functionality with normal receptors by fusion with the *Xenopus laevis* erythrocyte system (Strulovici et al., 1984). Figure 9 shows the results obtained. To the right are shown SDS gels of the receptor purified by affinity chromatography from control and desensitized cells. The receptor has been visualized by photoaffinity labeling. The altered mobility of the desensitized receptor appears to be due to its phosphorylation (Stadel et al., 1983; Sibley et al., 1983). As shown in the figure, there is a striking, approximately 50%, reduction in the ability of the desensitized receptors to stimulate the adenylate cyclase in the *X. laevis* acceptor cells (Strulovici et al., 1984). Comparable numbers of receptors were reconstituted and successfully fused in both the control and desensitized situations. Thus these data indicate that the desensitized and phosphorylated receptors are in fact functionally deficient as assessed in the reconstituted system.

Reconstitution of isolated receptors with purified guanine nucleotide regulatory proteins can also be used to assess the functionality of desensitized and/or covalently modified receptors. In order to test the hypothesis that the cyclic AMP-dependent protein kinase might be capable of regulating the function of the receptors we performed the experiments shown in Fig. 10. First, hamster lung β-adrenergic receptors were purified to homogeneity. When reconstituted in phospholipid vesicles together with the catalytic moiety of the cyclic AMP-dependent protein kinase and [^{32}P]ATP, 2 mol of phosphate could be inserted per mol of receptor (Benovic et al., 1985). The functionality of the phosphorylated receptor was then assessed by its ability

to mediate isoproterenol stimulation of the GTPase activity of N_s as shown in Fig. 10. It can be observed that a highly reproducible 25% reduction in the maximum isoproterenol-stimulated GTPase activity of N_s was observed. In a large number of experiments this result proved to be highly reproducible and highly statistically significant ($P < 0.001$). Although the 25% reduction in maximum functionality appears modest, it is precisely what would be expected. Thus, the avian erythrocytes show about a 50–60% desensitization when exposed to isoproterenol but only a 25–30% reduction when exposed to cyclic AMP (Stadel et al., 1981, 1983; Sibley et al., 1983). As shown in Fig. 10 this is closely paralleled by the reduction in functionality that can be achieved by directly phosphorylating the pure receptor with the cyclic AMP-dependent protein kinase. A likely supposition is that other protein kinases must participate in the phosphorylation reaction in order to observe the maximum 50–60% desensitization. Recent evidence with phorbol esters suggests that protein kinase *C* may also be capable of phosphorylating and densensitizing β-adrenergic receptors (Sibley et al., 1984; Kelleher et al., 1984; Nambi et al., 1985).

Future Perspectives

Studies of isolated receptors reconstituted with other components of the adenylate cyclase system are likely to occupy an increasingly important role in research on such systems in the years ahead. To date, only the β-adrenergic receptors have been purified sufficiently to attempt such investigations. However, it is reasonable to think that soon other purified receptors will become available and it will be interesting to compare their properties with the β-adrenergic receptors. Another goal will be to reconstitute a pure adenylate cyclase preparation together with receptors and guanine nucleotide regulatory proteins in order to study hormonal regulation in a system reconstituted from entirely pure components. Such systems should provide the basis for more complete understanding of the mechanisms involved in adenylate cyclase stimulation. Reconstitution of the inhibitory components, both receptors and N_i, should provide comparable information about the mechanisms of hormonal inhibition of the cyclase, especially for the currently unresolved question of which subunits of the heterotrimeric N_i protein are involved in the inhibitory pathway. Studies of newly discovered guanine nucleotide regulatory proteins such as N_o should serve to reveal the nature of the receptors to which they are physiologically coupled and thus the functional roles they play in normal biology. Applications to studies of physiological regulation such as desensitization as shown above are also likely to be important. Finally, as the genes for the individual components of the system are cloned, the gene products will become available in both substantial quantities as well as in structurally altered forms as a result of site-directed mutagenesis. Reconstituted systems will then be used to assess the functionality of various domains of the individual component proteins. Such studies should lead to new levels of understanding which relate the molecular structure of the receptors to their physiological functioning.

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