MOLECULAR PHARMACOLOGY, 15, 472-483

Adenylate Cyclase Assembled *In Vitro*: Cholera Toxin Substrates Determine Different Patterns of Regulation by Isoproterenol and Guanosine 5'-triphosphate

HARVEY R. KASLOW, ZVI FARFEL, GARY L. JOHNSON, AND HENRY R. BOURNE

Division of Clinical Pharmacology, Departments of Medicine and Pharmacology and the Cardiovascular Research Institute, University of California, San Francisco, San Francisco, California 94143

(Received October 2, 1978)
(Accepted December 6, 1978)

SUMMARY

KASLOW, HARVEY R., ZVI FARFEL, GARY L. JOHNSON & HENRY R. BOURNE. (1979) Adenylate cyclase assembled *in vitro*; cholera toxin substrates determine different patterns of regulation by isoproterenol and GTP. *Mol. Pharmacol.*, **15**, 472-483.

Genetic and biochemical evidence indicates that hormone-sensitive adenylate cyclase consists of at least three separable components: hormone receptor (R), a catalytic unit (C) that synthesizes cAMP from ATP, and one or more regulatory factors, which we have termed N, that are required for functional coupling of R and C and also for cyclase stimulation by guanyl nucleotides, NaF, and cholera toxin. The functional absence of N in a S49 lymphoma variant clone, *cyc*$^-$, allows *in vitro* assembly of hormone-sensitive adenylate cyclase, using *cyc*$^-$ membranes and N components in detergent extracts from membranes of other cells (Ross, E. M. and Gilman, A. G. *Proc. Nat. Acad. Sci., USA* 74,3715-3719, 1977). Such cyclase systems, assembled *in vitro* using N components from wild type S49 cells and turkey erythrocytes, are functionally distinguishable, each resembling—in responses to guanyl nucleotides and isoproterenol—the cyclase system from which the N component was derived. Thus, the N component determines certain functional characteristics of the response to guanine nucleotides and isoproterenol. Human erythrocyte membranes, which are virtually devoid of catalytic adenylate cyclase, also contain the functional N component. Donor extracts of human and turkey erythrocytes and S49 cells contain cholera toxin substrates, by two criteria: 1. The extracts transmit effects of toxin on donor membranes to adenylate cyclase assembled *in vitro* using *cyc*$^-$ membranes; 2. Incubation with toxin plus [^{32}P]NAD$^+$ specifically radiolabels substrates in each type of membrane, including a peptide of M_r = 42,000, common to all three. The results are consistent with the hypothesis that this peptide participates directly in multiple functions of N.

INTRODUCTION

Genetic and biochemical investigation has begun to unravel the molecular basis of hormonal stimulation of adenylate cyclase. In addition to hormone receptors and a catalytic component, the adenylate cyclase system of animal plasma membranes includes a third component (or class of components[1]) that serves to couple receptors

This work was supported by NIH grants HL 06285, GM 16496, and GM 00001, and NSF grant PCM 14397. GLJ is supported by NIH grant GM 06849. ZF is supported by a Merck International Fellowship in Clinical Pharmacology.

[1] No available evidence indicates whether the coupling function and the stimulation by guanyl nucleotide or fluoride ion require more than one class of molecules. Hereafter the factor will be referred to as

0026-895X/79/030472-12$02.00/0

and catalytic cyclase and also mediates activation of the enzyme by guanyl nucleotides, fluoride ion, and cholera toxin (1-6). Peptides associated with this coupling factor have been identified in mammalian (7) and avian (5, 8) membranes as substrates for specific radiolabeling by cholera toxin and [^{32}P]NAD$^+$.

How do catalytic cyclase and hormone receptors interact with the coupling factor? Some clues may be provided by comparison of two well-studied and phylogenetically separate hormone-sensitive cyclase systems, those of the mouse S49 lymphoma (9, 10) and the avian erythrocyte (11-16). The two systems are similar in that both are stimulated by β-adrenergic catecholamines and guanosine triphosphates, but several differences between the two systems have been documented: kinetic characteristics of adenylate cyclase stimulation by hormone and guanyl nucleotides (9, 12, 13), regulation by guanyl nucleotides of apparent affinity of β-receptors for binding agonists (9, 17), and the presence or absence of agonist-specific refractoriness of adenylate cyclase in intact cells (18, 19). If any one of these differences between systems can be assigned to a particular component—receptor, catalytic unit, or coupling factor—the function of that component and its interactions with the others will be better understood.

In this report we characterize and compare the properties of coupling factors and cholera toxin substrates of three membrane systems—S49 lymphoma, turkey erythrocyte, and human erythrocyte. Using a technique for *in vitro* assembly of hormone-sensitive adenylate cyclase developed by Ross and Gilman (1), we have assessed the function of heterologous coupling components of the cyclase system in a single membrane milieu. In this procedure, detergent extracts of donor membranes (e.g., turkey or human erythrocyte or wild type S49) are added to membranes of an S49 clonal variant line (20), termed *cyc*$^-$. The *cyc*$^-$ membranes contribute β-adrenergic receptors and catalytic units to the adenylate cyclase activity measured in the mixture, while the donor extracts supply the coupling factor, functionally lacking in *cyc*$^-$, that mediates cyclase stimulation by guanyl nucleotides and fluoride ion (1, 2). This coupling factor is associated with or identical to the membrane substrates of cholera toxin, also deficient in *cyc*$^-$, both functionally (6) and by the criterion of specific radiolabeling with [^{32}P]NAD$^+$ (7). The results indicate that the coupling factor, and not the β-adrenergic receptor or catalytic unit, determines kinetic characteristics of adenylate cyclase stimulation by hormone and guanyl nucleotides.

singular. However, the functions ascribed to the factor may in fact require more than one molecule in addition to receptor and catalytic units.

METHODS

Cell cultures. S49 mouse lymphoma cells were propagated as described (20) in Dulbecco's modified Eagle's medium containing 10% heat treated horse serum. Clones used were wild type (24.3.2) and *cyc*$^-$ (94.15.1, also termed ΔC$^-$ in other publications) (1-3, 20).

Cell membranes and extracts. S49 cell membranes were prepared by a modification (10) of the method of Ross *et al.* (9). Turkey erythrocytes were lysed in the presence of DNAse and membranes harvested as described (12). Human erythrocyte membranes were prepared using the same method with the omission of DNAse. Membranes were stored at −70°C. Erythrocyte membranes (2.5-3.5 mg protein/ml) were stored in 10 mM Tris[2] (pH 7.4), 0.3 M sucrose, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol. Before use, these membranes were suspended in 5-10 volumes of 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM 2-mercaptoethanol (TEM buffer), centrifuged (10 min at 27,000 × *g*), and resuspended in the desired volume of TEM

[2] The abbreviations used are: cAMP, cyclic 3',5'-adenosine monophosphate; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EGTA, ethyleneglycol-bis(β-aminoethylether)-N,N'-tetraacetic acid; GTPγS, guanosine-5'-O-(3-thiotriphosphate); Hepes, N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid; SDS, sodium dodecyl sulfate; TEM buffer, 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM 2-mercaptoethanol; Tris, tris(hydroxymethyl)aminomethane.

buffer. S49 membranes (4–8 mg protein/ml) were stored in 20 mM NaHepes (pH 8.0), 2 mM $MgCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 10% (w/v) glycerol. These membranes were simply diluted with TEM buffer to the desired volume.

Detergent extracts of S49 and turkey erythrocyte membranes (3–5 mg protein/ml) were prepared by adding Lubrol 12A9 to a final concentration of 0.7% (w/v), vigorously vortexing, and incubating overnight on ice. The mixture was then centrifuged (100,000 × *g*, 60 min) and the supernatant fraction was stored on ice or frozen rapidly and kept at −70°C. Extracts from human erythrocytes (2.5 mg protein/ml) were prepared in the same fashion, using 0.2% Lubrol.

Adenylate cyclase assembly and assay. Components of adenylate cyclase were assembled and assayed by adapting the method of Ross *et al.* (1). When Lubrol extracts were mixed with membranes of *cyc*$^-$ S49 cells, incubated on ice from 5–60 min and then assayed at 30°, the rate of cAMP synthesis increased during the first 5 to 15 min at 30° before a constant rate was achieved. We therefore first pipetted 10 μl of a Lubrol extract (containing Lubrol-extracted protein derived from 30–50 μg membrane protein) into a test tube sitting in ice, and began assembly by adding 70 μl of an assembly cocktail containing 30 μg *cyc*$^-$ membrane protein, vortexing, and incubating the mixture at 30° for 20 min. At this time, 20 μl of α[^{32}P]ATP (1.5 mM, 0.3–1 μCi) were added and the tube vortexed again. [^{32}P]cAMP synthesis under these conditions, in the presence of NaF (10 mM) or isoproterenol (100 μM) and GTPγS (100 μM) was constant for up to 90 min. After 40 min the assay was terminated and [^{32}P]-cAMP purified (21). The assembly cocktail contributed the following components (concentrations are those after dilution by the Lubrol extracts and the α[^{32}P]ATP, in a final volume of 100 μl): 50 mM NaHepes (pH 8.0), 6 mM $MgCl_2$, 0.2 mM EGTA, 2 mM 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin, 10 mM creatine phosphate, 10 U/ml creatine phosphokinase, 1 mM cAMP, 0.1 mM ATP, and S49 *cyc*$^-$ cell membranes, 0.3 mg protein/ml. Final ATP concentration was 0.4 mM. Effectors of adenylate cyclase included in the assembly cocktail when appropriate were: 10 mM NaF, 100 μM DL-isoproterenol, 100 μM GTP, 100 μM GTPγS, or 5 mM $MnCl_2$. When membranes were assayed without the addition of extracts, the same procedure was used, except that 10 μl of the membrane preparation were first pipetted into tubes, and the assembly cocktail did not contain *cyc*$^-$. The time course experiment employed the same concentrations of components in a large volume. After appropriate times, aliquots (100 μl) were withdrawn and [^{32}P]cAMP purified (21).

Cholera toxin treatment of membranes. Membranes (1.5–5 mg protein per ml) were incubated with 15 mM potassium phosphate (pH 7.4), 20 mM thymidine, 5 mM ADP-ribose, 20 mM arginine-HCl, 100 U/ml Trasylol, 100 μM GTP, 10 μg/ml cholera toxin (activated with 20 mM DTT [10]), and NAD^+. For labeling experiments [^{32}P]-NAD^+ was 10 μM (5–30 Ci/mmol); for other experiments NAD^+ was 1 mM. After addition of membranes, the mixture was incubated 20 min at 30°. For labeling experiments, the reaction was terminated by diluting with 10 volumes of ice cold 15 mM potassium phosphate (pH 7.4), centrifuging, resuspending in the same volume of phosphate buffer, and centrifuging once again. Pellets were suspended in 1% Lubrol and centrifuged after an overnight incubation on ice. To the supernatant were added SDS (1% final concentration) and 2-mercaptoethanol (5% final concentration). Samples were heated (60°, 60 min). After standing overnight at room temperature, the samples were electrophoretically analyzed (see below). For experiments involving activity determinations, the reaction ws terminated by adding 10 volumes ice cold TEM buffer and the membranes were washed twice by centrifugation. The membrane pellets were then suspended in TEM buffer and either assayed, extracted with Lubrol, or stored frozen.

Cholera toxin treatment of turkey erythrocyte membrane extracts mixed with S49 cyc$^-$ *membrane.* Lubrol extracts (5 ml) were applied to 2 ml Whatman DE-52 columns. The columns were washed with TEM

buffer, and 2 ml 0.5 M NaCl in TEM buffer were applied. The NaCl fraction contained component(s) that assembled a functional cyclase when incubated with S49 *cyc*$^-$ membranes and the assembly cocktail if Lubrol (0.016%) was present and ionic strength maintained (NaCl ≧ 100 mM). After this incubation, the reagents described above for cholera toxin reactions were added and this mixture incubated an additional 20 min at 30°. For labeling experiments, the mixtures were diluted, centrifuged, and processed as described above. For activity measurements, α[^{32}P]ATP plus appropriate effectors were added, and after 40 min at 30° [^{32}P]cAMP purified (21).

SDS polyacrylamide gel electrophoresis. Membranes (1.5–7 mg protein/ml) and Lubrol extracts were treated with SDS and 2-mercaptoethanol as described above. Discontinuous SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (22) as described by O'Farrell (23) using 10% acrylamide gels. After staining, destaining, and drying, X-ray film was exposed to the gels.

M_r determinations. The electrophoretic mobility of the peptide labeled by cholera toxin common to all membranes (except *cyc*$^-$) is the same as, or slightly greater than that of Band V of human erythrocyte membranes, with an estimated M_r = 41–43,000 ± 10% (24). Although a previous calibration of our gel system suggested M_r = 45,000 (7), here we assign M_r = 42,000 to the peptide, owing to its relation to Band V.

Materials. Heparinized turkey blood from female turkeys was purchased from the Willie Bird Turkey Farm, Petaluma, CA. Human erythrocytes in 0.38% sodium citrate were a gift from Dr. Paul Insel.

Compounds were of reagent grade. GTPγS was bought from Boehringer Mannheim. Creatine phosphate, creatine phosphokinase, DNase (type DN-100), ATP, GTP, Tris, Hepes, EDTA, EGTA, NAD$^+$, thymidine, ADP-ribose, and arginine-HCl were from Sigma Chemical Co. Trasylol was from Mobay Chemical Corporation.

SDS gel electrophoresis materials were from Bio-Rad. [^{32}P]NAD was synthesized from α[^{32}P]ATP (ICN) as described (7). Cholera toxin and sucrose (ultra pure) were from Schwarz-Mann. Lubrol 12A9 was a gift from ICI, Ltd. X-ray film (NS-2T and XR-2) was from Kodak.

DL-isoproterenol and L-norepinephrine were from Sigma Chemical Co. Practolol and L-propranolol were kind gifts of Ayerst Laboratories. L-epinephrine and L-isoproterenol were from Sterling-Winthrop.

RESULTS

Kinetic interaction of hormone and guanyl nucleotides. Several laboratories have studied catecholamine and guanyl nucleotide stimulation of adenylate cyclase in turkey erythrocyte (11–17) and wild type S49 membranes (9, 10). Isoproterenol, a potent β-adrenergic agonist, has been reported to exhibit a nearly absolute requirement for exogenous guanosine triphosphates to stimulate adenylate cyclase in both systems (9, 13, 15). In both types of membranes maximal activation occurs when isoproterenol is combined with a hydrolysis resistant guanosine triphosphate, such as guanylyl-5′-imidodiphosphate (Gpp(NH)p) or guanosine-5′-O-(3-thiotriphosphate) (GTPγS) (9, 13, 15). However, comparison of the time course of cAMP synthesis shows two clear differences between the two membranes (Fig. 1). First, GTP stimulates adenylate cyclase activity less effectively in turkey erythrocyte than

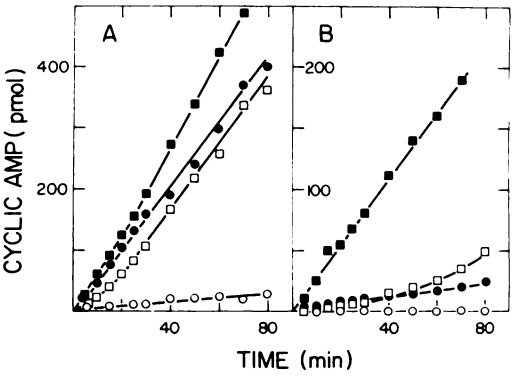

FIG. 1. *Time course of cAMP synthesis by (A) S49 wild type and (B) turkey erythrocyte membranes*

Effectors, 100 μM when present, were: ○ = GTP, □ = GTPγS, ● = GTP plus isoproterenol, ■ = GTPγS plus isoproterenol. Values represent amount of cAMP generated per 30 μg protein at 30°.

in S49 membranes, especially in the presence of isoproterenol (10% *vs.* 80% of maximal activity measured in the presence of isoproterenol plus GTPγS). Secondly, stimulation of cAMP synthesis by guanyl nucleotides shows a more stringent requirement for the simultaneous presence of isoproterenol in turkey erythrocyte than in S49 membranes. Thus, GTPγS alone activates cAMP synthesis much more slowly in the turkey system than in S49. As reported (9), isoproterenol affects the rate predominantly rather than the maximal extent of cyclase activation by hydrolysis-resistant GTP analogues. Measuring cAMP synthesis during a single time period from 20 to 60 min after addition of stimulating agents also demonstrates these differences (Table 1) (see METHODS).

In addition, as previously reported (10, 12), treatment of both types of membranes with cholera toxin makes the stimulatory actions of GTP resemble those of hydrolysis-resistant analogues. Although toxin treatment makes S49 membranes respond to GTP alone, toxin-treated turkey erythrocyte membranes continue to show a relatively more stringent requirement for the simultaneous presence of isoproterenol (Table 1). This direct comparison of the interaction of guanyl nucleotides and isoproterenol in the adenylate cyclase systems confirms previous studies of S49 and turkey erythrocyte membranes.

In vitro *assembly of adenylate cyclase.* Ross and Gilman (1) reported that mixing *cyc*⁻ "recipient" membranes with detergent-solubilized "donor" extracts from wild type S49 membranes leads to assembly of adenylate cyclase activity that can be stimulated by isoproterenol, guanyl nucleotides and fluoride ion. Donor extracts and recipient membranes, tested separately, showed little or no response to these effectors[3].

TABLE 1

Adenylate cyclase activity of wild type S49 and turkey erythrocyte membranes

Membranes were or were not treated with cholera toxin and assayed as described in METHODS.

Cholera toxin treatment	S49		Turkey Erythrocyte	
	−	+	−	+
Additions[a]	Relative activity[b]			
GTP	0.08	0.57	0.01	0.09
GTP + isoproterenol	0.80	0.89	0.11	0.62
GTPγS	0.86	0.86	0.38	0.38
NaF	0.77	0.39	0.18	0.14
GTPγS + isoproterenol	(234)	(257)	(109)	(105)[c]

[a] Additions were 100 μM, except NaF = 10 mM.

[b] Activity is relative to that in the presence of GTPγS plus isoproterenol.

[c] Values in parentheses represent pmol cAMP per min per mg membrane protein measured as described in METHODS. All values are means of duplicate experiments. The range of variability was no greater than 10% for any value.

These workers presented evidence (1-3) indicating that the *cyc*⁻ membrane contributes β-adrenergic receptors and catalytic adenylate cyclase to such mixtures, while donor extracts contribute one or more coupling or regulatory elements. In addition, assembly of cyclase activity responsive to isoproterenol requires an intact *cyc*⁻ membrane[4] (1). Which characteristics of the newly assembled cyclase system derive from regulatory (donor) elements, and which are derived from the membrane milieu and components contributed by *cyc*⁻? Certain characteristics of guanyl nucleotide and isoproterenol stimulation of cyclase are conferred by the regulatory factor in donor extracts (Table 2). The heterologous adenylate cyclase assembled from turkey erythrocyte donor extracts and *cyc*⁻ membranes synthesizes cAMP at maximal rates (stimulated by isoproterenol plus GTPγS), comparable to those seen in the homologous assembly (using wild type S49 extracts). However, regulation of adenylate cyclase in the heterologous assembly more

[3] Adenylate cyclase activity in *cyc*⁻ membranes is virtually undetectable in response to any of these effectors. Under the conditions used in the assembly experiments reported here (see METHODS), GTPγS and NaF cause barely detectable stimulation of adenylate cyclase activity in detergent solubilized extracts of wild type S49 or turkey erythrocyte membranes, while isoproterenol has no effect. In no case did the detergent extract show more than 10% of the activity measured after mixing it with *cyc*⁻ membranes (results not shown).

[4] G. L. Johnson, unpublished observations.

TABLE 2

Adenylate cyclase assembled in vitro

Donor extract[a]—Source—Cholera toxin treatment	S49 Wild Type		Turkey Erythrocyte		Human Erythrocyte	
	−	+	−	+	−	+
Additions[b]	*Relative activity*[c]					
GTP	0.04	0.71	0.02	0.20	0.02	0.85
GTP + isoproterenol	0.30	1.30	0.11	1.53	0.27	1.08
GTPγS	0.65	0.88	0.24	0.67	0.67	0.98
NaF	0.56	0.38	0.86	0.09	0.68	0.42
GTPγS + isoproterenol	(108)	(103)	(60)	(119)	(279)	(107)[d]

[a] Lubrol 12A9 extracts of donor membranes prepared and mixed with S49 *cyc*⁻ membranes as described in METHODS. Donor membranes were or were not treated with cholera toxin and NAD^+ before extraction with detergent.

[b] Additions were 100 μM except NaF, which was 10 mM.

[c] Activity is relative to that in the presence of GTPγS and isoproterenol.

[d] Values in parentheses represent pmol cAMP synthesized per min per mg *cyc*⁻ protein. Each value or fractional value shown is the mean of 5 or 6 experiments.

closely resembles that seen in turkey erythrocyte membranes than that of wild type S49 membranes or of the homologous adenylate cyclase assembled using S49 donor extracts (compare Fig. 1 and Table 1 with Table 2).

First, in the presence of isoproterenol GTP is relatively less effective as a stimulator of adenylate cyclase in the heterologous than in the homologous enzyme assembly (11% and 30%, respectively, of maximal cAMP synthesis). Second, stimulation of cAMP synthesis by GTP and GTPγS more stringently requires isoproterenol in both the heterologous enzyme system and turkey erythrocyte membranes than in S49. The persistence of these two differences between the donor membranes through solubilization with detergent and assembly with *cyc*⁻ membranes suggests intrinsic differences between specific regulatory components of the two cyclase systems.

Fluoride ion. In our hands NaF stimulates adenylate cyclase less effectively than isoproterenol plus GTPγS in turkey erythrocyte membranes and its effectiveness is not much reduced by treatment with cholera toxin and NAD^+ (Table 1). Other workers have reported that NaF stimulates cAMP synthesis to an extent comparable to that seen with isoproterenol plus GTPγS and that cholera toxin treatment markedly reduces the stimulatory effect of fluoride (12). No reason for these discrepancies is apparent, and both findings were reproducible in multiple independently prepared membrane samples in our laboratory.

It is also difficult to interpret the fact that NaF stimulates adenylate cyclase even better in the system assembled from turkey erythrocyte donor extract and *cyc*⁻ than in the homologously assembled enzyme (Table 2). The relative effects of NaF stimulation in the intact membranes do not parallel those seen in the enzyme systems assembled *in vitro*. Several possible explanations could account for these discrepancies, including: 1. Detergent in the assembly mixture depresses isoproterenol stimulation more than that of fluoride ion, changing their relative effectiveness[5]; 2. Detergent solubilization may alter regulatory components so that NaF stimulation is more effective; 3. A component contributed by *cyc*⁻ partially determines the relative effectiveness of NaF.

Nonetheless, cholera toxin treatment of turkey erythrocyte membranes before detergent extraction markedly reduces NaF stimulation of the heterologously assembled enzyme (Table 2). Toxin produces a similar, but less extensive, decrease in NaF stimulation of S49 membranes (Table 1) and of the homologously assembled enzyme (Table 2). Thus the effects of cholera toxin on both GTP and fluoride regulation are

[5] This appears to be the case with wild type S49 membranes; H. R. Kaslow, unpublished observations.

transmitted to the cyclase assembled *in vitro*.

In summary, these results indicate that donor detergent extracts from both S49 and turkey erythrocyte membranes contain a regulatory component of adenylate cyclase that can be altered by cholera toxin and that determines the pattern of cyclase responsiveness to isoproternenol, guanyl nucleotides, and —at least in part—fluoride ion.

Classification of β-adrenergic receptors. The relative potencies of adrenergic agonists in stimulating cAMP synthesis by turkey erythrocyte membranes follow the pattern described for the β_1-subclass of receptor (isoproterenol > norepinephrine > epinephrine, in the ratio of 1:0.46:0.13, respectively), while the S49 membrane shows a β_2 pattern (isoproterenol > epinephrine ≫ norepinephrine, in the ratio of 1:0.23:0.015, respectively). Practolol, a relatively selective β_1-adrenergic antagonist, is 5-fold more potent as an inhibitor of isoproterenol stimulation of adenylate cyclase in turkey erythrocyte membranes than in S49 (results not shown).

The coupling factor supplied in donor extracts does not determine the pharmacological subclass of β-adrenergic receptor in the enzyme assembled *in vitro*, however. As defined by rank order of agonist potency and relative resistance to antagonism by the β_1-adrenergic blocker, practolol, the enzyme assembled *in vitro* using the turkey erythrocyte donor extract shows a β_2 pattern of responsiveness to catecholamines (isoproterenol > epinephrine > norepinephrine, in the ratio of 1:0.14:0.011, respectively), like that of S49 and unlike that of the turkey erythrocyte. Thus the patterns of guanyl nucleotide responsiveness and pharmacological subclassification of β-adrenergic receptors are determined by physically separable components.

Donor extracts from human erythrocytes. Unlike avian erythrocytes, human erythrocytes are almost devoid of adenylate cyclase activity (25). Our human erythrocyte membrane preparation synthesizes less than 4 pmol cAMP per min-mg protein (in the presence of NaF or isoproterenol plus GTPγS), a rate that is less than 4% of that observed in wild type S49 or turkey erythrocyte membranes. Mn^{2+} ion (5 mM), reported to stimulate adenylate cyclase activity in several tissues and cell types, including *cyc*$^-$ (3, 26), does not stimulate cAMP synthesis by human erythrocyte membranes or detergent extracts (result not shown). This result suggests that the catalytic unit of adenylate cyclase is functionally deficient in human erythrocytes.

Nonetheless, detergent extracts of human erythrocyte membranes contain a component (or components) that can assemble adenylate cyclase activity if mixed with *cyc*$^-$ membranes (Table 2). In its responsiveness to GTP and its requirement for isoproterenol, the cyclase assembled using human erythrocyte extracts resembles that assembled from S49, rather than the turkey erythrocyte. Human erythrocyte membranes also contain a functional cholera toxin substrate, as shown by the increased responsiveness to GTP of adenylate cyclase assembled *in vitro* from extracts of toxin-treated human erythrocyte membranes (Table 2).

Identification of cholera toxin substrates. What molecules in donor extracts transfer the effects of cholera toxin to adenylate cyclase assembled *in vitro*? Wild type S49 membranes contain multiple peptides specifically radiolabeled by incubation with cholera toxin and [^{32}P]NAD$^+$: A single band of M_r = 42,000 and a doublet of M_r = 52–53,000 are seen on polyacrylamide electrophoresis in SDS; these bands are deficient in *cyc*$^-$ (7; Figs. 2 and 3). Specific cholera toxin substrates can also be identified in extracts of human and turkey erythrocyte membranes (Figs. 2 and 3).

Human erythrocyte membranes contain only one peptide (M_r = 42,000) specifically radiolabeled with cholera toxin and [^{32}P]-NAD$^+$ (Fig. 2). Background (nonspecific) labeling in the absence of toxin is quite low.

In turkey erythrocyte membranes, however, radiolabeling of material in the absence of toxin frustrated our initial attempts at identifying specific toxin substrates (Fig. 3). In order to reduce this background, an alternative procedure was developed. The regulatory factor in detergent extracts of turkey erythrocyte mem-

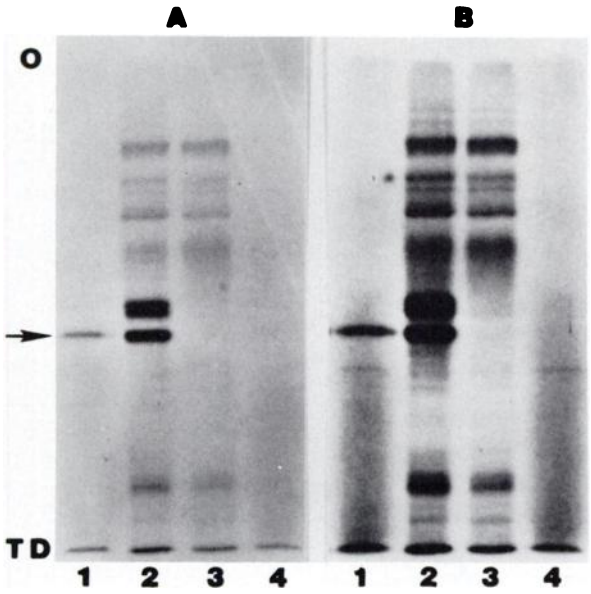

FIG. 2. *Autoradiograms of an SDS slab gel electrophoresis of Lubrol extracts of membranes incubated with [^{32}P]NAD$^+$ either with or without cholera toxin*

A = short exposure; B = long exposure. 1 = human erythrocyte with cholera toxin; 2 = S49 wild type with cholera toxin; 3 = S49 wild type alone; 4 = human erythrocyte alone. O = origin, TD = position of tracking dye, arrow = M_r 42,000.

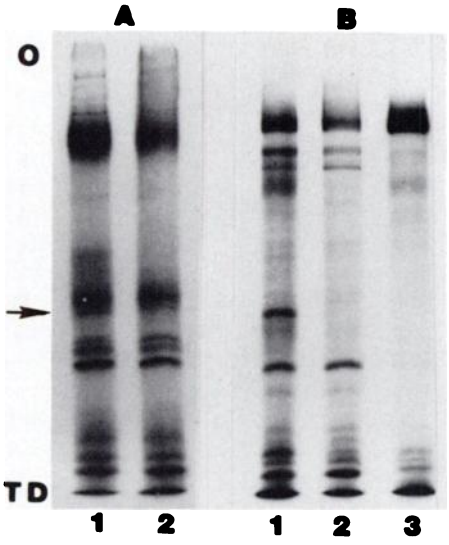

FIG. 3. *Autoradiograms of the SDS slab gel electrophoresis of Lubrol extracts from membranes incubated with [^{32}P]NAD$^+$ with or without cholera toxin and DEAE extract*

(A) turkey erythrocyte membranes incubated with [^{32}P]NAD$^+$ either (1) with or (2) without cholera toxin; and (B) S49 *cyc*$^-$ membranes incubated with [^{32}P]-NAD$^+$ plus (1) DEAE turkey erythrocyte membrane fraction and cholera toxin, (2) DEAE turkey erythrocyte membrane fraction, and (3) cholera toxin. O = origin, TD = position of tracking dye, arrow = M_r 42,000.

branes was adsorbed to DEAE-cellulose, eluted with salt, and then added to *cyc*$^-$ membranes (see METHODS). Adenylate cyclase in the resulting mixture was similar, in its pattern of response to isoproterenol and guanyl nucleotides (not shown), to that assembled using the original detergent extract (Table 2). In addition, treatment of this mixture (turkey erythrocyte DEAE extract plus *cyc*$^-$ membranes) with cholera toxin and NAD$^+$ increased GTP stimulation of adenylate cyclase (not shown) in the presence of isoproterenol, just as if the turkey erythrocyte membranes had been treated with toxin before assembly of the cyclase *in vitro*.

The ability of the DEAE extract to confer toxin-sensitivity on *cyc*$^-$ membranes made the experiment depicted in Fig. 3 feasible. Treatment of the mixture of DEAE extract and *cyc*$^-$ membrane with toxin and [^{32}P]-NAD$^+$ produces specific radiolabeling of a

peptide band of M_r = 42,000. As reported previously (7), toxin causes no specific radiolabeling of cyc^- membranes alone (Fig. 3). A simple interpretation of this result is that the turkey erythrocyte extract contributed a specific toxin substrate.

The radiolabeling experiments with erythrocyte membranes indicate that a single toxin substrate in donor extracts is sufficient to transfer the effects of cholera toxin observed in the *in vitro* assemblies of adenylate cyclase.

DISCUSSION

Current evidence (1-6) indicates that the transduction of an external hormonal signal into intracellular cAMP synthesis requires a minimum of three distinct membrane components: Hormone receptors (R), catalytic adenylate cyclase (C), and a coupling component, termed N (6) because it mediates effects of guanyl nucleotides. Although more than one molecular species may play a role in performing functions ascribed to N, one of these is the class of membrane peptides that can serve as substrates for ADP-ribosylation by cholera toxin (5, 7, 8). The absence of N in cyc^- S49 lymphoma cells, both functionally (6) and as a substrate for cholera toxin (7), allows the *in vitro* assembly of hormone-sensitive adenylate cyclase systems by combining cyc^- membranes with N components derived from other cells (1-3, 6). The present report compares N components and cholera toxin substrates of mouse S49 cells with those of turkey and human erythrocytes. The results establish that N determines certain functional differences between adenylate cyclase systems of different cell types. The results also show that the ability of N to interact with other components of adenylate cyclase has been conserved in the divergent evolution of birds and mammals, as was implied by earlier studies (27) of heterokaryons constructed from turkey erythrocytes and murine cells.

Turkey erythrocyte compared with S49. The adenylate cyclase system of turkey erythrocytes differs from that of S49 cells in: 1. Responding to β-adrenergic agents in a pattern characteristic of β_1- rather than β_2-adrenergic receptors; 2. Decreased relative stimulation by GTP compared to GTPγS; 3. More stringently requiring isoproterenol for stimulation by guanyl nucleotides. Our results indicate that the difference between β_1 and β_2 receptors is not due to interactions of similar R with different N components. In contrast, differing responsiveness to GTP and requirements for isoproterenol reflect intrinsic properties of N components in the two cyclase systems, rather than their interactions with R, C, or the membrane environment.

Recent kinetic studies of the turkey erythrocyte cyclase system suggest possible reasons for these differences. Cassel and Selinger described an isoproterenol-stimulated GTPase activity in turkey erythrocyte membranes (15), kinetically related to the activation of adenylate cyclase by isoproterenol and GTP (13) and blocked by treatment with cholera toxin (12). They proposed that the binding of GTP activates adenylate cyclase and that hydrolysis of GTP to GDP reduces cyclase activity; isoproterenol activates cAMP synthesis by promoting the replacement of GDP at the nucleotide site by GTP (14). Thus the increased stimulation of cyclase by GTP in S49 relative to that of turkey erythrocyte membranes (Fig. 1, Table 1) may be due to less rapid hydrolysis of the nucleoside triphosphate in S49. If so, N components in donor extracts confer characteristically different rates of GTP hydrolysis on adenylate cyclase assembled *in vitro*, since the effects of GTP in mixtures of each donor extract with cyc^- resemble those observed in the respective donor membranes (Tables 1 and 2).

Two additional observations suggest that isoproterenol stimulates a lower rate of GTP hydrolysis in wild type S49 membranes: 1. Under conditions in which isoproterenol-stimulated GTPase was easily measured in turkey erythrocyte membranes (i.e., background GTPase activity in S49 membranes is appropriately low), no stimulation of GTPase by isoproterenol in S49 membranes is detected.[6] 2. Cassel and

[6] H. R. Bourne, unpublished observations.

Selinger reported that the increased rate of cAMP synthesis in turkey erythrocyte membranes exposed to GTP and epinephrine persists for 4 sec after addition of propranolol (13); we confirmed this result but found that catecholamine-stimulated rates of cAMP synthesis by S49 membranes persist for 25–30 sec after propranolol.[7] Both these findings are consistent with a slower rate of hydrolysis of GTP at the activating nucleotide site in S49 membranes.

Activation of cAMP synthesis by guanyl nucleotides shows a more stringent requirement for isoproterenol in turkey erythrocyte than in S49 membranes (Fig. 1, Table 1). Cassel *et al.* recently reported that isoproterenol releases GDP from turkey erythrocyte membranes, and proposed that the catecholamine makes the guanyl nucleotide site more accessible to guanyl nucleotides (14). We have confirmed this result.[8] It is possible that the nucleotide binding site of the S49 N component is more accessible to guanyl nucleotides in the absence of isoproterenol, although the hormone analogue can still promote more rapid binding of GTP and GTPγS. If so, the N components of donor extracts determine similar relative accessibilities of nucleotide binding sites in the *in vitro* assemblies of adenylate cyclase, since the characteristic requirement for isoproterenol persists in the mixture of turkey erythrocyte donor extract and cyc^-.

Cholera toxin substrates. Peptides that are specifically radiolabeled by cholera toxin in wild type S49 membranes are not detected in cyc^- membranes which are also functionally deficient in N (1–6, 7; Figs. 2 and 3). Toxin also catalyzes transfer of the ADP-ribose moiety of [^{32}P]NAD$^+$ to a single peptide in pigeon erythrocyte membranes (5, 8). This ADP-ribosylated protein was partially purified on a guanyl nucleotide column; addition of the partially purified protein fraction to column effluent fractions containing catalytic adenylate cyclase increased stimulation of the cyclase by guanyl nucleotide (5). This genetic and biochemical evidence indicates that cholera toxin substrates are closely linked or identical to the cyclase component, which we have termed N, that mediates stimulation of cAMP synthesis by guanyl nucleotides and fluoride ion.

What is the relation of cholera toxin substrates to coupling of hormone receptors and cAMP synthesis? The hypothesis that these substrates participate directly in coupling of R to C is consistent with all the evidence presented here: By the criterion of specific radiolabeling with [^{32}P]NAD$^+$, donor extracts derived from wild type S49 and turkey and human erythrocytes contain such substrates, which are deficient in cyc^- (7, Figs. 2 and 3). N components in each of these donor extracts contribute to functionally distinguishable assemblies of isoproterenol-stimulated adenylate cyclase activity in combination with cyc^- membranes, and in each case the characteristic effect of toxin treatment, enhanced sensitivity to GTP, is transmitted to the assembled enzyme (Table 2).

The data do not rule out the possibility that other molecules in donor extracts, in addition to the toxin substrates, may be necessary for coupling R to C. Receptors for β-adrenergic amines and prostaglandins are functionally uncoupled from cAMP synthesis in variant S49 clones of the UNC phenotype (28), and UNC membranes exhibit radiolabeled toxin substrates similar in apparent molecular weight to those of wild type S49 cells (7). Determination of the number of different molecular species that comprise N, R, and C will ultimately require their complete purification and reconstitution into a functioning hormone-sensitive adenylate cyclase.

The polyacrylamide gel patterns of [^{32}P]NAD$^+$-labeled toxin substrates do impose certain constraints on the possible molecular complexity of coupling receptors to cAMP synthesis. Gel patterns indicate single labeled peptide bands (M_r = 42,000) in human, turkey (Figs. 2 and 3), and pigeon erythrocytes (5, 8). Membranes of wild type S49 and rat hepatoma (HTC-4) cells exhibit a similar labeled band and an additional doublet (M_r = 52–53,000 [7; Fig. 2]). Our data indicate that donor extracts containing a single toxin substrate, supplied by either human or turkey erythrocytes, can confer

[7] Z. Farfel, unpublished observations.

[8] H. R. Kaslow, unpublished observations.

on cyc^- membranes sensitivity to isoproterenol, guanyl nucleotides and NaF. Thus, these activities do not require the additional toxin substrates (presumably the doublet of M_r = 52–53,000) present in wild type S49. Furthermore, these additional S49 peptides do not account for the differences between S49 and turkey erythrocyte membranes observed in activation by isoproterenol and GTP, because human erythrocyte extracts, containing a single toxin substrate, combine with cyc^- to assemble an enzyme similar to that assembled from S49 (Table 2).

No biological function has been assigned to the additional toxin-labeled peptides of wild-type S49 membranes. Because these peptides are not observed in cyc^-, they could be metabolic precursors of the smaller peptide and/or play a role in other processes absent in cyc^-, such as agonist-specific refractoriness (18) or guanyl nucleotide regulation of the affinity of β-adrenergic receptors for binding catecholamine agonists (9).

Human erythrocytes as a source of N. The presence in human erythrocyte membranes of a functional N component of adenylate cyclase and a substrate for cholera toxin (Table 2, Fig. 2) is significant for three reasons.

1. Despite low adenylate cyclase activity (25, and confirmed in our study), β-adrenergic amines or prostaglandins affect several properties of human erythrocytes, including deformability (25), membrane potential (29), and phosphorylation of membrane proteins (30). Marginally detectable cAMP elevations in these cells may mediate the effects of hormones. Alternatively, membrane molecules that perform the functions ascribed to N in coupling receptors to cAMP synthesis may also couple receptors to other effector enzymes.

2. Because so little cyclase activity is present, we can conclude that cholera toxin can effectively modify the M_r = 42,000 peptide in the virtual absence of catalytic cyclase activity.

3. Compared to plasma membranes of most animal cells, those of the human erythrocyte are more easily separated from other cellular components and more extensively studied. These characteristics should make the human erythrocyte a useful source for purification and characterization of the N component of hormone-regulated adenylate cyclase.

ACKNOWLEDGMENT

We thank Ms. Mary Gleason for expert technical assistance.

REFERENCES

1. Ross, E. M. & Gilman, A. G. (1977) Reconstitution of catecholamine-sensitive adenylate cyclase activity; interaction of solubilized components with receptor-replete membranes. *Proc. Nat. Acad. Sci., USA*, **74,** 3715–3719.
2. Ross, E. M. & Gilman, A. G. (1977) Resolution of some components of adenylate cyclase necessary for catalytic activity. *J. Biol. Chem.*, **252,** 6966–6969.
3. Ross, E. M., Howlett, A. C., Ferguson, K. M. & Gilman, A. G. (1978) Reconstitution of hormone-sensitive adenylate cyclase activity with resolved components of the enzyme. *J. Biol. Chem.*, **253,** 6401–6412.
4. Pfeuffer, T. (1977) GTP-binding proteins in membranes and the control of adenylate cyclase activity. *J. Biol. Chem.*, **252,** 7224–7234.
5. Cassel, D. & Pfeuffer, T. (1978) Mechanism of cholera toxin action: Covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. *Proc. Nat. Acad. Sci., USA*, **75,** 2669–2673.
6. Johnson, G. L., Kaslow, H. R. & Bourne, H. R. (1978) Reconstitution of cholera toxin-activated adenylate cyclase. *Proc. Nat. Acad. Sci., USA*, **75,** 3113–3117.
7. Johnson, G. L., Kaslow, H. R. & Bourne, H. R. (1978) Genetic evidence that cholera toxin substrates are regulatory components of adenylate cyclase. *J. Biol. Chem.*, **253,** 7120–7123.
8. Gill, D. M. & Meren, R. (1978) ADP-ribosylation of membrane proteins catalyzed by cholera toxin; basis of the activation of adenylate cyclase. *Proc. Nat. Acad. Sci., USA*, **75,** 3050–3054.
9. Ross, E. M., Maguire, M. E., Sturgill, T. W., Biltonen, R. L. & Gilman, A. G. (1977) Relationship between the beta-adrenergic receptor and adenylate cyclase. *J. Biol. Chem.*, **252,** 2761–2775.
10. Johnson, G. L. & Bourne, H. R. (1977) Influence of cholera toxin on the regulation of adenylate cyclase by GTP. *Biochem. Biophys. Res. Comm.*, **78,** 792–798.
11. Spiegel, A. M. & Aurbach, G. D. (1974) Binding of 5′-guanylyl-imidophosphate to turkey erythrocyte membranes and effects on beta-adrenergic

activated adenylate cyclase. *J. Biol. Chem.*, **249**, 7630–7636.

12. Cassel, D. & Selinger, Z. (1977) Mechanism of adenylate cyclase activation by cholera toxin; inhibition of GTP hydrolysis at the regulatory site. *Proc. Nat. Acad. Sci., USA*, **74**, 3307–3311.
13. Cassel, D., Levkovitz, H. & Selinger, Z. (1977) The regulatory GTPase cycle of turkey erythrocyte adenylate cyclase. *J. Cyclic Nucleo. Res.*, **3**, 393–406.
14. Cassel, D. & Selinger, Z. (1978) Mechanism of adenylate cyclase activation through the beta-adrenergic receptor; catecholamine-induced displacement of bound GDP by GTP. *Proc. Nat. Acad. Sci., USA*, **75**, 4155–4159.
15. Cassel, D. & Selinger, Z. (1976) Catecholamine stimulated GTPase activity in turkey erythrocyte membranes. *Biochim. Biophys. Acta*, **452**, 538–551.
16. Cassel, D. & Selinger, Z. (1977) Catecholamine-induced release of [^{3}H]Gpp(NH)p from turkey erythrocyte adenylate cyclase. *J. Cyclic Nucleo. Res.*, **3**, 11–22.
17. Brown, E. M., Fedak, S. A., Woodard, C. J., Aurbach, G. D. & Rodbard, D. (1976) Beta-adrenergic receptor interactions: Direct comparison of receptor interaction and biological activity. *J. Biol. Chem.*, **251**, 1239–1246.
18. Shear, M., Insel, P. A., Melmon, K. L. & Coffino, P. (1976) Agonist-specific refractoriness induced by isoproterenol. *J. Biol. Chem.*, **251**, 7572–7576.
19. Hanski, E. & Levitzki, A. (1978) The absence of desensitization in the beta-adrenergic receptors of turkey reticulocytes and erythrocytes and its possible origin. *Life Sci.*, **22**, 53–60.
20. Bourne, H. R., Coffino, P. & Tomkins, G. M. (1975) Selection of a variant lymphoma cell deficient in adenylate cyclase. *Science*, **187**, 950–952.
21. Salomon, Y., Londos, C. & Rodbell, M. (1974) A highly sensitive adenylate cyclase assay. *Anal. Biochem.*, **58**, 541–548.
22. Laemmli, V. K. (1975) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
23. O'Farrell, P. Z., Gold, L. M. & Huang, W. M. (1973) The identification of pre-replicative bacteriophage T4 protein. *J. Biol. Chem.*, **248**, 5499–5501.
24. Steck, T. L. (1974) The organization of proteins in the human red blood cell membrane. *J. Cell Biol.*, **62**, 1–19.
25. Rasmussen, H., Lake, W. & Allen, J. E. (1975) The effect of catecholamines and prostaglandins upon human and rat erythrocytes. *Biochim. Biophys. Acta*, **411**, 68–73.
26. Naya-Vigne, J., Johnson, G. L., Bourne, H. R. & Coffino, P. (1978) Complementation analysis of hormone-sensitive adenylate cyclase. *Nature*, **272**, 1720–1722.
27. Orly, J. & Schramm, M. (1976) Coupling of catecholamine receptor from one cell with adenylate cyclase from another cell by cell fusion. *Proc. Nat. Acad. Sci., USA*, **73**, 4410–4414.
28. Haga, T., Ross, E. M., Anderson, H. J. & Gilman, A. G. (1977) Adenylate cyclase permanently uncoupled from hormone receptors in a novel variant of S49 mouse lymphoma cells. *Proc. Nat. Acad. Sci., USA*, **74**, 2016–2020.
29. Lavie, E. & Sonenberg, M. (1978) Depolarization of mature human erythrocytes induced by catecholamine hormones. *Endocrinology*, **102 (suppl.)**, 273.
30. Tsukamoto, T. & Sonenberg, M. (1977) Catecholamine regulation of human erythrocyte membrane phosphorylation. *Endocrinology*, **100 (suppl.)**, 285.