When rat adipocyte membranes had been labeled with \(^{3}H\)GTP in the presence of a \(\beta\)-adrenergic agonist, the subsequent \(^{3}H\)GDP release was stimulated by \(\beta\)-agonists or agonists (e.g. glucagon and secretin) of other "activatory" receptors involved in activation of adenylate cyclase, but was not stimulated by agonists (e.g. prostaglandin E\(_{2}\) and adenosine) of "inhibitory" receptors involved in cyclase inhibition. On the contrary, agonists of inhibitory receptors were effective in stimulating GDP release from hamster adipocyte membranes that had been labeled with GTP in the presence of a \(\alpha\)-adrenergic agonist, but an activatory receptor agonist such as isoproterenol was not. Thus, the guanine nucleotide regulatory protein (N\(_{i}\)) involved in adenylate cyclase inhibition is an entity distinct from the regulatory protein (N\(_{s}\)) involved in cyclase activation, and multiple activatory or inhibitory receptors are coupled to a respective common pool of N\(_{i}\) or N\(_{s}\). Preactivated cholera toxin added together with NAD enhanced GDP release from rat adipocyte membranes prelabeled with isoproterenol but was without effect on the release from hamster adipocyte membranes that had been labeled with an \(\alpha\)-agonist. In sharp contrast, the active subunit of islet-activating protein, pertussis toxin, failed to alter GDP release from the former membrane but completely abolished inhibitory agonist-induced stimulation of GDP release from the latter membrane preparation in the presence of NAD. Thus, the site of action of cholera toxin is N\(_{i}\), while that of islet-activating protein is N\(_{s}\). The function of N\(_{s}\) to communicate between inhibitory receptors and adenylate cyclase was lost when it was ADP-ribosylated by islet-activating protein.

Substantial evidence has been so far presented for the pivotal role of the guanine nucleotide regulatory protein in receptor-mediated activation of adenylate cyclase (1-4). Stimulation of the membrane receptor by an agonist leads to its association with the nucleotide regulatory protein releasing GDP from the binding sites on the regulatory protein in exchange for GTP. This is the "turnon" reaction, since the GTP-bound form of the regulatory protein activates the catalytic unit of adenylate cyclase. Hydrolysis of GTP to GDP on the nucleotide regulatory protein terminates this activation as the "turnoff" reaction. Thus, receptor agonists such as peptide hormones, neurotransmitters, and autacoids appear to stimulate cAMP production by promoting the exchange of bound GDP with the activator GTP on the nucleotide regulatory protein. Cassel and Selinger (5) were the first to demonstrate directly the hormone-stimulated release of GDP from the cell membrane. Agents that interact directly with the guanine nucleotide regulatory protein should also exert an influence on the GTP-GDP exchange reaction. Such is a case with cholera toxin. The release of GDP from erythrocyte membranes was actually stimulated by the toxin (6).

Apart from these "activatory" receptors, there are several types of receptor that should be termed "inhibitory" in the sense that they are linked to the cyclase catalytic unit conversely in an inhibitory fashion. This receptor-linked inhibition of adenylate cyclase is also mediated by the guanine nucleotide regulatory protein occasionally referred to as N\(_{i}\), which appears to be a different entity from the nucleotide regulatory protein (N\(_{s}\)) involved in activation of cyclase. N\(_{i}\) is very similar to N\(_{s}\) in its behavior as a coupling protein between the receptor and the catalytic unit of cyclase. For instance, Michel and Lefkowitz (7) have recently reported that epinephrine, via \(\alpha\)-adrenergic receptors, promotes the release of Gpp(NH)p from human platelet membranes and that there is a significant correlation between an adrenergic agonist's intrinsic activity for adenylate cyclase inhibition and its intrinsic activity for formation of \(^{3}H\)Gpp(NH)p release. The GTP-GDP exchange on N\(_{i}\) also appears to result from agonist binding to inhibitory receptors and is the mechanism involved in the receptor-mediated inhibition of adenylate cyclase.

Thus, agonist-induced release of an isotopically labeled guanine nucleotide from membranes may reflect coupling of the receptor to the nucleotide regulatory protein, regardless of whether it mediates activation or inhibition of adenylate cyclase.

Islet-activating protein (8), pertussis toxin, is a unique modifier of the receptor-adenylate cyclase system (9, 10). It is an oligomeric protein consisting of an A-protomer and a B-protomer (11). The A-protomer of IAP is an enzyme, ADP-ribosyltransferase (12), and penetrates across the cell membrane as a result of the binding of the holotoxin, via the B-protomer moiety, to the surface of the membrane (13). After reaching to the site of the holotoxin inside the membrane, the A-protomer of IAP catalyzes ADP-ribosylation of a M\(_{r}\) = 41,000 peptide.
membrane protein (14) which is presumably one of the subunits of the guanine nucleotide regulatory protein (15). Later studies have shown that the negative signal transduction via N, from the receptor to the adenylate cyclase enzyme was abolished, but that the positive signal transduction via N, was not, by IAP-catalyzed ADP-ribosylation of the membrane protein (16-18). This will give full explanation to the earlier observations that the inhibitory receptor-mediated decreases in the cellular content of cAMP were attenuated or abolished by the prior treatment of the cell with IAP (19-22). Possibly, as cholera toxin is a modifer of N, so is IAP an uncoupler of N, (16-18).

The purpose of the present paper is to study coupling conditions between the pools of the receptors and the guanine nucleotide regulatory proteins by means of an assay of agonist-induced \( ^{3}H \)GDP release from rat and hamster adipocyte membranes. The adipocyte membranes are suitable for this purpose, since there are multiple receptors that are linked to adenylate cyclase in an activatory or inhibitory fashion in the membranes. The effect of IAP on agonist-induced GDP release was compared with that of cholera toxin. The results will show that N, is the cholera toxin-sensitive site whereas N, is the site of the action of IAP.

**EXPERIMENTAL PROCEDURES**

**Materials**—IAP prepared from the 2-day culture supernatant of Bordetella pertussis (8) was exposed to 5 M urea for 4 days at 4°C to liberate its A-protomer (an enzymatically active peptide), which was then isolated by chromatography on a column of carboxymethyl-Sepharose CL-6B (11). Cholera toxin, purchased from Chemo-Sero Therapeutic Research Institute (Kumamoto, Japan), was preactivated shortly before use in occlusion. At 37°C for 20 min with 50 mM dithiothreitol in 0.1 M phosphate buffer (pH 7.4), a procedure that releases an enzymatically active A, fragment (23). Prostaglandin E\(_2\), propranolol, salbutamol, and procaterol were kind gifts from Tokaysha Pharmaceutical Company (Tokushima, Japan). Reagents for radioimmunoassay of cAMP were obtained from a Yamasa cyclic AMP assay kit which was generously donated by Yamasa Shoyu Co. (Chiba, Japan). Collagenase was a product (Type I) of Worthington. \( ^{3}H \)GTP (10-15 Ci/mmol) was obtained from New Engand Nuclear; polyethylenimine cellulose thin layer chromatography sheets (Polygram Cell 300PE1) were from Macherey-Nagel Co. The sources of other reagents were described in the previous papers. 

**Adipocyte Membrane Preparation**—Fat cells were isolated from male rats of the Donryu strain (body weight 200-220 g) by the method developed with 1.5 M LiCl. The spots corresponding to authentic GTP, GDP, GMP, and GDP in 200 pl of ice-cold Buffer B; 1 ml of the supernatant obtained by a 3-min centrifugation at 25,000 \( \times g \) was counted for tritium.

The release of \( ^{3}H \)GDP from rat adipocyte membranes induced by the bacterial toxin (Fig. 4 and Table IV) was assayed differently in the following respect. The assay mixture consisted of 0.1 M phosphate buffer (pH 7.4), 5 mM MgCl\(_2\), 1 mM EGTA, and 0.3 mM GTP; further additions of preactivated cholera toxin (or preactivated adenylate cyclase) or of other nucletides in Table III and supplemented with or without receptor antagonists or their antagonists as indicated in the tables and figures. The mixture was incubated at 30°C for 5 min (otherwise as in Fig. 1) in the case of rat adipocyte membranes or for 5 min (or otherwise as in Fig. 5) in the case of hamster adipocyte membranes. The assay was terminated by the addition of 1 ml of ice-cold Buffer B; 1 ml of the supernatant obtained by a 3-min centrifugation at 25,000 \( \times g \) was counted for tritium.

**Release of \( ^{3}H \)GDP from rat adipocyte membranes induced by the bacterial toxin (Fig. 4 and Table IV) was assayed differently in the following respect. The assay mixture consisted of 0.1 M phosphate buffer (pH 7.4), 5 mM MgCl\(_2\), 1 mM EGTA, and 0.3 mM GTP; further additions of preactivated cholera toxin (or preactivated adenylate cyclase) or of other nucletides in Table III and supplemented with or without receptor antagonists or their antagonists as indicated in the tables and figures. The mixture was incubated at 30°C for 5 min (otherwise as in Fig. 1) in the case of rat adipocyte membranes or for 5 min (or otherwise as in Fig. 5) in the case of hamster adipocyte membranes. The assay was terminated by the addition of 1 ml of ice-cold Buffer B; 1 ml of the supernatant obtained by a 3-min centrifugation at 25,000 \( \times g \) was counted for tritium.

**Thin Layer Chromatography of Guanine Nucleotides Released**—A 1-ml aliquot of the supernatant of the reaction mixture of the \( ^{3}H \)GDP release assay, obtained after incubation as described above, was washed twice in 0.1 M NaCl and taken of 10-15 pg of protein. A 1-ml aliquot was applied to a thin layer plate of polyethyleneimine cellulose and developed with 1.5 M LiCl. The spots corresponding to authentic GTP, GDP, and GMP were scraped and counted for tritium.

**Adenylyl Cyclase Assay**—This was conducted as described previously (9). Washed membranes (10-15 pg of protein) were incubated for 10 min at 30°C in 150 pl of Buffer B further supplemented with 1 mM EGTA, 0.3 mM dithiothreitol, 0.3 mM 3-isobutyl-1-methylxanthine, 6.7 mM phosphocreatine, 30 units/ml of creatine phosphokinase, and 1 mg/ml of bovine serum albumin. The reaction mixture was further supplemented with 0.1 M NaCl in the case of hamster adipocyte membranes. Other additions including 0.1 mM GTP were present where indicated. Cyclic AMP generated during incubation was measured by a sensitive radioimmunoassay method (27). Protein was determined according to the method of Lowry et al. (27) using bovine serum albumin as standard.

**Data Presentation**—Radioactivities bound to membranes after the above procedures of \( ^{3}H \)GTP loading and washing varied somewhat among different batches of membrane preparation. The absolute value for \( ^{3}H \) release was dependent on the bound \( ^{3}H \) and agreed well within a batch of membranes. Despite such variability of the absolute value for the bound \( ^{3}H \), similar results were obtained for effects of additions on \( ^{3}H \) release by experiments repeated two or three times under the same conditions. Data recorded in the present study are the mean values from duplicate observations, generally agreeing within 5%, in representative experiments, unless otherwise specified.
RESULTS

Isoproterenol-induced Exchange of Guanine Nucleotides—
Rat adipocyte membranes that had bound [3H]GTP in the presence of isoproterenol released [3H]-labeled guanine nucleotides at a relatively constant rate during the subsequent release assay (Fig. 1). The addition of isoproterenol produced an immediate increase in the rate of release, which returned to the basal rate within 3–4 min. Isoproterenol-induced release of [3H]-labeled nucleotides was dependent on the presence of the catecholamine in the first "loading" step; no nucleotide was released unless the preincubation of membranes with [3H]GTP was conducted in the presence of isoproterenol (Table I). More [3H]GTP bound to membranes in the presence of isoproterenol, and about a half of this extra bound nucleotides was released in the second "unloading" step with nonradioactive GTP and isoproterenol (Table I). Chromatographic separation of released nucleotides as described under "Experimental Procedures" revealed that greater than 90% of the guanine nucleotides released specifically in response to isoproterenol was GDP (data not shown).

Isoproterenol failed to release [3H]GDP from labeled rat adipocyte membranes in the absence of unlabeled guanine nucleotides (Table II). The presence of either GTP or Gpp(NH)p allowed the largest [3H]GDP release. Guanosine 5'-O-(2-thiodiphosphate) was also effective but GMP and ATP were almost ineffective in this regard. The combined addition of both GTP and Gpp(NH)p caused [3H]GDP release not exceeding that induced by either alone.

The data in Fig. 1 and Tables I and II are thus in accordance with the current model of coupling of membrane receptors with the guanine nucleotide regulatory protein. Conceivably, the membrane preparation from rat adipocytes contains bound GDP, and introduction of [3H]GTP in the presence of isoproterenol in the first loading step may reflect β-adrenoceptor-mediated exchange of GTP–GDP at the regulatory site. [3H]GTP thus bound to the regulatory site is apparently hydrolyzed to [3H]GDP. This is released in exchange for added Gpp(NH)p or GTP during the subsequent release assay again as a result of stimulation of coupled β-adrenergic receptors by isoproterenol.

TABLE I
Isoproterenol requirement during [3H]GTP labeling of rat adipocyte membranes

| Addition during labeling | Membrane bound | Release from membranes | Increase by isoproterenol |
|--------------------------|----------------|------------------------|--------------------------|
|                          |                | -isoproterenol | +isoproterenol | cpm/assay tube |
| No addition | 3413 (3.25) | 611 | 638 | 27 (0.03) |
| Isoproterenol | 4229 (4.03) | 887 | 1275 | 388 (0.37) |

* Values in parentheses are picomoles/mg of protein.

Fig. 1. Time course of isoproterenol-induced [3H]GDP release from rat adipocyte membranes. Rat adipocyte membranes were labeled with 0.5 μM [3H]GTP in the presence of isoproterenol under the standard conditions described under "Experimental Procedures." The labeled membranes were then submitted to the [3H]GDP release assay with 0.3 mM Gpp(NH)p in the presence (●) or absence (○) of isoproterenol for the time indicated on the abscissa. The isoproterenol-induced release of [3H]GDP (△) was also plotted as the difference between ● and ○.

TABLE II
Guanine nucleotide requirement for isoproterenol-induced [3H]GDP release from rat adipocyte membranes

| Unlabeled nucleotide addition | [3H]GDP release by isoproterenol pmol/mg protein |
|------------------------------|-----------------------------------------------|
| None                        | 0.01                                           |
| Gpp(NH)p                    | 0.38                                           |
| GTP                         | 0.28                                           |
| GTP + Gpp(NH)p              | 0.34                                           |
| GDP•S                       | 0.38                                           |
| GMP                         | 0.11                                           |
| ATP                         | 0.09                                           |

Fig. 2. Release of [3H]GDP from rat adipocyte membranes in response to increasing concentrations of various β-adrenergic agonists. Rat adipocyte membranes labeled with 0.5 μM [3H]GTP as in Fig. 1 were then submitted to the [3H]GDP release assay with 0.3 mM Gpp(NH)p for 5 min in the presence of isoproterenol (○), norepinephrine (△), epinephrine (□), salbutamol (○), or procaterol (△). The assay mixture containing isoproterenol was further supplemented with 50 μM propranolol (×). Agonist-induced release is plotted as a function of the agonist concentrations.

In Table II, the GDP released in response to the addition of isoproterenol was concentration-dependent and antagonized by propranolol in a competitive manner (Fig. 2, left). Evidently, stimulation of β-adrenergic receptors is involved in the [3H]GDP release observed. Dose-response curves for a series of β-adrenergic agonists were constructed for [3H]GDP release (Fig. 2, right). The potency for nucleotide release was not so markedly different among these agonists; the half-maximally effective concentration was 0.5–2 μM for the five agonists tested. Their efficacies were, however, variable. Salbutamol and procaterol released less nucleotide than did isoproterenol.
or epinephrine when tested at their maximally effective concentrations.

GTP-dependent activation of adenylate cyclase by increasing concentrations of these agonists was also studied with the same rat adipocyte membrane preparation (Fig. 3). Isoproterenol, epinephrine, and norepinephrine were equally potent in adenylate cyclase activation and in stimulating [3H]GTP release, but salbutamol and procaterol were 1 order of magnitude less potent in adenylate cyclase activation than in promoting nucleotide release. As regards their intrinsic activities, the agonists which promote more [3H]GDP release also show greater activity in adenylate cyclase activation. Indeed, the correlation between an agonist's intrinsic activity for activation of adenylate cyclase and its intrinsic activity for [3H]GDP release was high significant ($r = 0.997, p < 0.001$; a correlation plot is not shown but compare Figs. 3 and 2, right).

Effects of Other Receptor Agonists on [3H]GDP Release from Rat Adipocyte Membranes—The [3H]GDP release from rat adipocyte membranes that had been labeled with [3H]GTP in the presence of isoproterenol was promoted not only by isoproterenol itself but also by glucagon or secretin, although less nucleotide was released in the latter case (Table III). Fluoride was without effect on [3H]GDP release by itself, nor did it affect the isoproterenol-induced release. Likewise, neither $N^6$-methyladenosine nor prostaglandin E$_1$ caused [3H]GDP release. Isoproterenol was as effective in the presence of the adenosine analogue as in its absence. Glucagon or secretin activates adenylate cyclase in the presence of relatively low concentrations of GTP, whereas $N^6$-methyladenosine and prostaglandin E$_1$ inhibit the cyclase at any effective GTP concentration in rat adipocyte membranes (data not shown but see our previous report (17)). Thus, it is suggested from the data in Table III that guanine nucleotides bound to rat adipocyte membranes as a result of stimulation of $\beta$-adrenergic receptors can be released by agonists of the activatory receptors but not by agonists of the inhibitory receptors.

Promotion of [3H]GDP Release from Rat Adipocyte Membranes by Cholera Toxin, but Not by IAP—Rat adipocyte membranes were first labeled with [3H]GTP in the presence of various concentrations of isoproterenol and salbutamol, or procaterol. The control activity estimated without any agonist and the maximal activity estimated with the saturating concentration (400 $\mu$M) of isoproterenol were 25 and 590 pmol of cAMP/mg/min, respectively. Both values were used to calculate agonist-stimulated adenylate cyclase activity expressed as a percentage of the maximal activity.

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![Fig. 3. Concentration-dependent activation of membrane adenylate cyclase by various $\beta$-adrenergic agonists. Rat adipocyte membranes were assayed for adenylate cyclase activity as described under "Experimental Procedures" with 0.1 $\mu$M GTP in the presence of various concentrations of isoproterenol (O), epinephrine (A), norepinephrine (O), salbutamol (Q), or procaterol (A). The control activity estimated without any agonist and the maximal activity estimated with the saturating concentration (400 $\mu$M) of isoproterenol were 25 and 590 pmol of cAMP/mg/min, respectively. Both values were used to calculate agonist-stimulated adenylate cyclase activity expressed as a percentage of the maximal activity.](http://www.jbc.org/Downloaded from)

### Table III

| Addition                      | [3H]GDP release induced (pmol/mg protein) |
|-------------------------------|------------------------------------------|
| Isoproterenol (100 $\mu$M)    | 0.24                                     |
| Glucagon (1 $\mu$M)           | 0.15                                     |
| Secretin (0.1 $\mu$M)         | 0.11                                     |
| NaF (10 mM)                   | 0.0                                      |
| Isoproterenol + NaF           | 0.21                                     |
| $N^6$-Methyladenosine (100 $\mu$M) | 0.0                                      |
| Isoproterenol + $N^6$-methyladenosine (2 $\mu$M) | 0.25                                     |
| Prostaglandin E$_1$ (10 $\mu$M) | 0.0                                      |

### Table IV

Effects of cholera toxin and A-protomer of IAP on [3H]GDP release from rat adipocyte membranes

| Addition                      | [3H]GDP release induced (pmol/mg protein) |
|-------------------------------|------------------------------------------|
| NAD (100 $\mu$M)              | 0.03                                     |
| Preactivated cholera toxin (25 $\mu$g/ml) | 0.04                                     |
| NAD + preactivated cholera toxin | 0.25                                     |
| A-protomer of IAP (25 $\mu$g/ml) | 0.00                                     |
| NAD + A-protomer of IAP       | 0.01                                     |

![Fig. 4. Time course of cholera toxin-induced [3H]GDP release from rat adipocyte membranes. Rat adipocyte membranes labeled with 0.5 $\mu$M [3H]GTP in the presence of isoproterenol were then submitted to the [3H]GDP release assay with 0.3 mM Gpp(NH)p in the presence of the additions shown below. Data show the increment in [3H]GDP release due to addition.](http://www.jbc.org/Downloaded from)
GDP Release from Membranes Susceptible to Choleragen or IAP

in the next series of experiments, rat adipocyte membranes were incubated with the preactivated choleragen or the A-protomer of IAP in the presence of NAD. Cholera toxin-specific or IAP-specific substrate proteins of rat adipocyte membranes were fully ADP-ribosylated under these conditions (17). These toxin-treated membranes were then labeled with [3H]GTP in the presence of isoproterenol and submitted to the [3H]GDP release assay as shown in Table V. Neither isoproterenol nor glucagon was effective in promoting [3H]GDP release from cholera toxin-treated membranes, despite their normal activities to stimulate nucleotide release from IAP-treated membranes. In the IAP-treated membranes from which [3H]GDP was released in response to isoproterenol or glucagon, the amount of nucleotide released by combined addition of both agonists was essentially the same as the amount released by isoproterenol alone (Table V).

Labeling of Hamster Adipocyte Membranes with [3H]GTP via α-Adrenergic Receptors and the Subsequent Release of [3H]GDP As a Result of Stimulation of the Same Receptors—Hamster adipocyte membranes are characterized by the occurrence of α-adrenergic receptors that are involved in inhibition of adenylyl cyclase (29-31). In fact, adenylyl cyclase of our membrane preparation of hamster adipocytes was inhibited via α-adrenergic receptors in the presence of 0.1 M NaCl and 0.1-100 M GTP (data not shown). This inhibition was totally abolished by prior treatment of the membranes with the A-protomer of IAP under the conditions as described under "Experimental Procedures" which resulted in ADP-ribosylation of the M, = 41,000 membrane protein (data not shown) just as had been observed with rat adipocytes (17).

[3H]GDP release dependent on α-adrenergic receptors was then studied with the membrane preparation from hamster adipocytes. Membranes first incubated with [3H]GTP were again incubated with nonlabeled GTP in the fresh medium to measure the release of the once bound labeled nucleotides in exchange for the unlabeled GTP. The addition of epinephrine, with either an α- or a β-adrenergic antagonist, to the medium in the unloading step did not stimulate [3H] release significantly unless the medium for the first loading procedure was supplemented with epinephrine (Table VI). In this experiment, propranolol, a β-adrenergic antagonist, was combined with epinephrine during the loading procedure to allow the catecholamine to stimulate α-adrenergic receptors selectively. The release of labeled nucleotides from these membranes was promoted by epinephrine more strongly in the presence of propranolol than in the presence of yohimbine, an α-antagonist. Chromatographic analysis performed as described above revealed that GDP comprised most (over 90%) of the nucleotides released from hamster adipocyte membranes by α-adrenergic agonists.

TABLE V

| Pretreatment of membranes | [3H]GDP released by |
|--------------------------|---------------------|
|                          | Isoproterenol | Glucagon | Isoproterenol plus glucagon |
| Cholera toxin A-protomer of IAP | 0.07 | 0.05 | ND |
| A-protomer of IAP | 0.34 | 0.21 | 0.36 |

* ND, not determined.

TABLE VI

| [3H]GDP released with | Membrane-bound | Epinephrine plus |
|-----------------------|----------------|-----------------|
|                        | Yohimbine | Propranolol |
| [3H]GTP labeling | | |
| None | 1345 | 386 | 404 | 400 | 0.08 | 0.06 |
| Epinephrine plus | 1572 | 548 | 575 | 678 | 0.12 | 0.59 |
| Propranolol | | |

4 Release induced by epinephrine in the presence of yohimbine, i.e. via β-adrenergic receptors.

4 Release induced by epinephrine in the presence of propranolol, i.e. via α-adrenergic receptors.

Fig. 5 (left). Time course of α-adrenergic receptor-stimulated [3H]GDP release from hamster adipocyte membranes. Hamster adipocyte membranes were labeled with 2 μM [3H]GTP in the presence of epinephrine plus propranolol as described under "Experimental Procedures." Labeled membranes were then submitted to the [3H]GDP release assay in the presence of 0.3 mM GTP and 50 μM propranolol with (○) or without (□) 100 μM epinephrine for 3 min. The assay mixture containing clonidine was further supplemented with 50 μM yohimbine (x).

Fig. 6 (right). Concentration-dependent release of [3H]GDP from hamster adipocyte membranes by β-adrenergic agonists. Hamster adipocyte membranes labeled with 2 μM [3H]GTP as in Fig. 5 were submitted to the [3H]GDP release assay in the presence of 0.3 mM GTP and 50 μM propranolol with various concentrations of epinephrine (○), norepinephrine (△), or clonidine (□) for 3 min. The assay mixture containing clonidine was further supplemented with 50 μM yohimbine (x).
GDP Release from Membranes Susceptible to Cholera or IAP

nephrine (Fig. 6), in accordance with its rather weak action to inhibit adenylate cyclase of hamster adipocyte membranes (data not shown). This activity of clonidine was totally blocked by the simultaneous addition of yohimbine to the assay medium.

Thus, the results in Table VI and Figs. 5 and 6 indicate that [3H]GTP bound to hamster adipocyte membranes as a result of stimulation of α-adrenergic receptors was hydrolyzed to [3H]GDP and then released upon the second stimulation of the same type of receptors.

[3H]GDP Release from Hamster Adipocyte Membranes Differentially Dependent on Activatory and Inhibitory Receptors—Hamster adipocyte membranes were labeled with [3H]GTP in the presence of either isoproterenol, a β-adrenergic agonist, or epinephrine plus propranolol (Table VII). [3H]GDP release from the membranes labeled with isoproterenol was promoted by the same agonist. It was also promoted by epinephrine plus yohimbine. But, epinephrine plus propranolol was less effective, and prostaglandin E, or nicotinic acid was essentially without effect in this regard.

The relative efficiencies of these receptor agonists to promote [3H]GDP release were quite different when hamster adipocyte membranes had been labeled with [3H]GTP via α-adrenergic receptors, i.e. in the presence of epinephrine plus propranolol. From membranes labeled in this way, more [3H]GDP was released by epinephrine in the presence of propranolol than in the presence of yohimbine in accordance with Table VI. A significant amount of [3H]GDP was released from the same membranes upon the addition of prostaglandin E, or nicotinic acid. Moreover, isoproterenol was less effective in promoting [3H]GDP release from membranes labeled with [3H]GTP via α-receptors than from membranes labeled via β-receptors stimulated by this selective agonist.

Prostaglandin E, and nicotinic acid, together with α-adrenergic agonists, are known to inhibit adenylate cyclase in hamster adipocyte membranes via the respective membrane receptors (29–32). The results in Table VII are, therefore, considered to show that stimulation of these inhibitory receptors located on hamster adipocyte membranes results in the GDP-GTP exchange at the binding sites of a common pool of N, which is not directly linked with activatory receptors such as β-adrenergic ones.

Inhibitory Effect of the A-protomer of IAP on α-Adrenocep-
tor-mediated GDP Release from Hamster Adipocyte Mem-
bribes—Hamster adipocyte membranes that had been treated with the active subunit of cholera toxin or IAP were used to study the effect of these bacterial toxins on the α-adrenergic receptor-mediated [3H]GDP release (Table VIII). Both [3H]GTP loading and the subsequent [3H]GDP unloading were carried out in the presence of an α-adrenergic agonist. [3H]GDP was released in response to epinephrine or norepinephrine from cholera toxin-treated membranes in an amount essentially the same as, or only slightly smaller than, that from control membranes not treated with the toxin. Thus, cholera toxin failed to exert any significant influence on the α-receptor-mediated [3H]GDP release from hamster adipocyte membranes.

In sharp contrast, no [3H]GDP was released at all under the same conditions from membranes that had been treated with the A-protomer of IAP (Table VIII). This result can be interpreted to reflect a lack of an α-receptor-mediated GTP-GDP exchange in membranes ADP-ribosylated by the A-protomer of IAP. Alternatively, [3H]GDP may have been released spontaneously from these IAP-treated membranes during the preceding 5-min washing procedure.

A more acute effect of the A-protomer of IAP was then studied in Table IX. In experiments shown in this table, membranes were first labeled with [3H]GTP via α-adrenergic receptors stimulated by epinephrine plus propranolol, and the medium for a 5-min washing of thus labeled membranes was supplemented with the A-protomer of IAP and NAD. This IAP treatment of [3H]GTP-labeled membranes also abolished the subsequent [3H]GDP release in response to epinephrine (plus propranolol) as well as to prostaglandin E, (Table IX). The direct addition of the A-protomer of IAP (together with

| Pretreatment of membranes with | [3H]GDP release induced by | pmol/mg protein |
|-------------------------------|---------------------------|----------------|
| Epinephrine | Norepinephrine |
| None | 0.53 | 0.51 |
| Cholera toxin | 0.42 | 0.34 |
| A-protomer of IAP | -0.03 | 0.01 |

| Effect of the A-protomer of IAP on [3H]GDP release from hamster adipocyte membranes prelabeled with [3H]GTP via α-adrenergic receptors |
|---------------------------------------------------------------|
| Hamster adipocyte membranes were labeled with 2 μM [3H]GTP in the presence of isoproterenol or epinephrine plus propranolol as described under "Experimental Procedures." Labeled membranes were then submitted to the [3H]GDP release assay in the presence of 0.3 mM GTP, 10 μM propranolol, and 1200 μM epinephrine or 100 μM norepinephrine. Radioactivity bound to the labeled membranes was 1284, 1262, and 1146 cpm/assay for nontreated, cholera toxin-treated, and the A-protomer-treated membranes, respectively, immediately prior to the [3H]GDP release assay. |

| [3H]GDP labeling with | [3H]GDP release induced by | pmol/mg protein |
|----------------------|---------------------------|----------------|
| Epinephrine plus propranolol | - | 0.65 |
| Epinephrine plus propranolol | + | 0.08 |

| Table VII |
|---------------------|
| Receptor-mediated release of [3H]GDP from hamster adipocyte membranes labeled with [3H]GTP via α- or β-adrenergic receptors |
| Hamster adipocyte membranes were labeled with 2 μM [3H]GTP in the presence of isoproterenol or epinephrine plus propranolol as described above. Labeled membranes were then submitted to the [3H]GDP release assay in the presence of 0.3 mM GTP with the additions shown below. The concentrations of additions to the [3H]GDP release assay were 100 μM isoproterenol, 100 μM epinephrine, 10 μM yohimbine, 10 μM propranolol, 10 μM prostaglandin E. (PGE) E, and 100 μM nicotinic acid. |

| [3H]GDP labeling with | [3H]GDP release induced by | pmol/mg protein |
|----------------------|---------------------------|----------------|
| Epinephrine plus propranolol | - | 0.65 |
| Epinephrine plus propranolol | + | 0.08 |
NAD) to the medium for the [3H]GDP release assay did not accelerate spontaneous [3H]GDP release (data not shown). Thus, it can be concluded that stimulation of inhibitory receptors by their respective agonists did not promote GTP-GDP exchange on Ni of hamster adipocyte membranes provided the M, = 41,000 protein of the membranes was ADP-ribosylated by the A-protomer of IAP.

DISCUSSION

A β-adrenergic agonist promotes the release of [3H]GDP from the cell membrane that has been labeled with [3H]GTP in the presence of the same agonist. This β-agonist-induced [3H]GDP release has been studied with membranes of turkey (5, 6, 33) and frog (33) erythrocytes. It occurs only in the presence of nonlabeled guanine nucleotides and is correlated with the concurrent change in adenylate cyclase activity. Probably, the [3H]GTP loading results from the exchange with the endogenously bound GDP, and the [3H]GDP unloading reflects again the exchange with the newly added GTP or Gpp(NH)p, both at the regulatory sites under the influence of coupled receptors stimulated by added β-agonists. The [3H]GDP release assay appears, therefore, to afford a good index for the receptor-mediated GTP-GDP exchange on the guanine nucleotide regulatory protein which is responsible for activation of the adenylate cyclase catalytic unit. More direct evidence has recently been provided for the involvement of the nucleotide regulatory protein in β-receptor-mediated [3H]GDP release in turkey erythrocytes (28).

The present study has confirmed the previous reports about properties of β-adrenergic receptor-linked [3H]GDP release with the use of the membrane preparation from another cell type, rat adipocytes. Isoproterenol was required for the first [3H]GTP labeling, and Gpp(NH)p (or GTP) was essential for the subsequent isoproterenol-induced release of [3H]GDP. The intrinsic activities of several β-adrenergic agonists for [3H]GDP release correlated well with their respective intrinsic activities for adenylate cyclase activation. Thus, the guanine nucleotide regulatory protein (N,) that is responsible for adenylate cyclase activation is functionally coupled with β-adrenergic receptors in membranes prepared from rat white adipocytes.

The β-adrenergic receptors of rat adipocytes should be classified as a β,-type because norepinephrine was as potent as epinephrine in stimulating [3H]GDP release and in activating adenylate cyclase. Salbutamol and procaterol, β,-selective agonists, displayed lower intrinsic activities as compared with epinephrine or norepinephrine. This is in agreement with our previous findings that the intrinsic activities of β2-adrenergic agents were much lower than those of the nonselective agonists for increasing the cellular cAMP content of the rat cardiac cells, the typical cells exhibiting β2-selectivity (34). In addition, β2-selective agonists exhibited much less potency than nonselective or β1-selective agonists in activating adenylate cyclase (Fig. 3). Although an underlying mechanism is unknown, this would be another reason why these β2-agonists give rise to cAMP accumulation only slightly in adipocytes.

In rat adipocyte membranes, there are more than one kind of receptors that are linked with N,. Glucagon or secretion, like isoproterenol, was capable of promoting [3H]GDP release from the nucleotide pool that had been labeled via β-adrenoceptors. Isoproterenol and glucagon together released no more GDP than did isoproterenol alone. These three kinds of receptors have access to the entire pool of nucleotide regulatory proteins as previously reported with β-adrenergic and prostaglandin E, receptors in frog erythrocytes (33). It is likely, therefore, that all the classes of activatory receptors stimulated by different agonists are linked to a common pool of the guanine nucleotide regulatory protein (N,) that is responsible for adenylate cyclase activation. The amount of [3H]GDP released in response to the glucagon or secretin addition was considerably smaller than the amount released by isoproterenol. It might reflect a variable efficiency of the coupling to N, among these individual classes of receptors.

In sharp contrast, neither Nmethyladenosine nor prostaglandin E1, effective in promoting [3H]GDP release from rat adipocyte membranes that had been labeled with [3H] GTP in the presence of isoproterenol. These agonists inhibit adenylate cyclase in rat adipocyte membranes in a GTP-dependent manner (17). The guanine nucleotide pool labeled with [3H]GTP as a result of stimulation of coupled β-adrenergic receptors appears not to be linked with these inhibitory receptors which are responsible for adenylate cyclase inhibition. Michel and Lefkowitz (7) were the first to report about the inhibitory receptor-linked guanine nucleotide release. For this purpose, they used membranes of human platelets. In this cell type, membranes trapped Gpp(NH)p in the absence of any receptor agonist and subsequently released it in response not only to prostaglandin E1, an activatory receptor agonist in this cell type, but also to an agonist of the inhibitory α2-adrenergic receptors. Since more [3H]Gpp(NH)p was released in the presence of both an α-agonist and prostaglandin E1, than in the presence of either alone, they concluded that activatory and inhibitory receptors are linked to different pools of guanine nucleotides. Distinct from these platelet membranes, rat or hamster adipocyte membranes released no detectable amount of [3H]GDP in response to an agonist of activatory (or inhibitory) receptors unless [3H]GTP loading had been performed with a β- or an α-agonist. Conceivably, the endogenously bound GDP will not be displaced by added [3H]GTP without stimulation of coupled receptors in adipocyte membranes. Thus, the nucleotide regulatory proteins appear to be coupled to activatory receptors (and inhibitory receptors, too, as discussed below) more "tightly" in membranes of rat and hamster adipocytes than in membranes of human platelets.

Along with these previous data, the present results are consistent with the idea that N, and N, are functionally different entities; both are linked selectively to the activatory and the inhibitory receptors, respectively, in membranes. Attempts have been unsuccessful to observe [3H]GDP release from rat adipocyte membranes in response to any agonist of inhibitory receptors. This is because no suitable high affinity antagonist is available for the adenosine or prostaglandin receptor despite stringent requirement for it to retain the 3H-labeled nucleotide bound during the washing procedure following [3H]GTP loading. This is the reason why we used hamster adipocyte membranes in experiments shown in the later part of this study. Hamster adipocyte possess α2-adrenergic inhibitory receptors (30, 35, 36) for which highly selective and high affinity antagonist are available.

A significant amount of [3H]GDP was released from hamster adipocyte membranes that had been labeled with [3H] GTP via stimulation of α-receptors only if the medium for the GDP release assay was supplemented with an α-agonist itself, prostaglandin E1, or nicotinic acid, agonists of other inhibitory receptors. A β-adrenergic agonist was much less effective than an α-agonist in this regard, indicating that α- and β-receptors are linked to different pools of the nucleotide regulatory proteins. Thus, multiple receptors are coupled to a common pool of N, that is an entity distinct from N, in hamster adipocyte membranes. Less GDP was released in
response to prostaglandin or nicotinic acid then in response to an \(\alpha\)-agonist, probably reflecting variable efficiency in receptor-N coupling, just as was observed with glucagon and secretin in GDP release from the rat cell membranes (see above).

One of the principal purposes of the present study is to compare the effects of two bacterial toxins on the \([^{3}H]GDP\) release assay. The effects of cholera toxin on rat adipocyte membranes were essentially the same as those previously reported with turkey erythrocyte membranes by Burns et al. (6). When rat adipocyte membranes that had been labeled with \([^{3}H]GTP\) in the presence of isoproterenol were subsequently ADP-ribosylated by cholera toxin, \([^{3}H]GDP\) was released spontaneously without addition of receptor agonists. Thus, cholera toxin interacted with N, and, like \(\beta\)-adrenergic agonists, increased the rate of GTP-GDP exchange at the regulatory sites. In contrast, \(\alpha\)-receptor-mediated \([^{3}H]GDP\) release from hamster adipocyte membranes was not significantly altered by cholera toxin treatment. Cholera toxin does not appear to interact with \(N_{\alpha}\) it interacts with \(N_{\beta}\) exclusively.

If rat adipocyte membranes were first treated with cholera toxin and then labeled with \([^{3}H]GTP\), much less \([^{3}H]GDP\) was subsequently released from the toxin-treated membranes than from nontreated membranes in response to receptor agonists. This effect of the cholera toxin pretreatment could be explained by the following ways. (a) The toxin-treated membranes are once labeled with \([^{3}H]GTP\), but the bound nucleotides are released during the subsequent washing procedure due to the toxin-induced decrease in the affinity of N, for guanine nucleotides. (b) \([^{3}H]GTP\) bound to the toxin-treated membranes survives the process of washing, but it is not converted to \([^{3}H]GDP\) because of inhibition of GTPase by the toxin. The rate of exchange of added Gpp(NH)p with \([^{3}H]GTP\) must be lower than the rate of exchange with \([^{3}H]GDP\) during the subsequent process of release assay. (c) The communication between activatory receptors and N, is somehow interfered with as a result of the toxin-catalyzed ADP-ribosylation of membranes. Hence, much less \([^{3}H]GTP\) binds to, and much less nucleotide is then released from, such toxin-treated membranes than control membranes in the presence of isoproterenol.

The mechanism (a) is likely, since it is consistent with the direct effect of the toxin, to cause \([^{3}H]GDP\) release from \([^{3}H]GTP\)-labeled membranes as shown in Table IV. Supports for the mechanism (c), i.e. the interference with receptor-N, coupling by cholera toxin, are as follows. (i) The basal or GTP-dependent adenylate cyclase activity was higher, but its response to isoproterenol was relatively smaller, in rat adipocyte membranes pretreated with cholera toxin than in nontreated control membranes (17). (ii) Guanine nucleotides negatively modulate the affinity of receptors for agonists in membranes of various cell types. This effect of guanine nucleotides is considered to reflect coupling of the receptors to guanine regulatory protein (1,3). The Gpp(NH)p-induced negative modulation of the agonist affinity was much less in magnitude in cholera toxin-treated membranes of rat heart cells than in nontreated membranes of the same cells.8 (iii) IAP-catalyzed ADP-ribosylation of the \(M_{r} = 41,000\) membrane protein (14,15) results in uncoupling of N, from inhibitory receptors in rat adipocyte membranes (17). ADP-ribosylation of N, might likewise lead to impaired communication between N, and activatory receptors. Further studies will be required for full elucidation as to how the function of N, is modified following ADP-ribosylation by cholera toxin.

The A-protomer (active component) of IAP is absolutely without effect on \([^{3}H]GDP\) release from rat adipocyte membranes that have been labeled with \([^{3}H]GTP\) in the presence of isoproterenol. According to the foregoing discussion that the \([^{3}H]GDP\) release under these conditions reflects the guanine nucleotide exchange reaction of N, the failure of the A-protomer of IAP to promote GDP release is in good agreement with our previous conclusion that IAP interacts directly with \(N_{\alpha}\), rather than \(N_{\beta}\), in adipocyte membranes (17). Direct evidence was then provided for an interaction of the A-protomer of IAP with \(N_{\alpha}\) with the use of hamster adipocyte membranes; the effects of an \(\alpha\)-agonist and prostaglandin E\(_{1}\) to promote GDP release were abolished by treatment of the membranes with the A-protomer of IAP. Thus, inhibitory receptors were uncoupled from \(N_{\alpha}\), in hamster adipocytes after IAP-specific ADP-ribosylation of one of the subunits of \(N_{\alpha}\), with a \(M_{r}\) value of 41,000 (37).

It has recently been reported that enkephalin-induced hydrolysis of GTP probably occurring on \(N_{\alpha}\) in membranes from NG108-15 cells was prevented by prior treatment of the cells with IAP (38). A similar inhibition of GTP hydrolysis was caused by IAP in membranes from rat C6 glioma cells (19). The inhibition by IAP of receptor-mediated GTP hydrolysis may have resulted from functional uncoupling of \(N_{\alpha}\) from inhibitory receptors as evidenced in the present study, although much remains to be solved as to how the function of \(N_{\alpha}\) is impaired by IAP-catalyzed ADP-ribosilation in a manner somewhat different from cholera-catalyzed ADP-ribosylation of \(N_{\beta}\).

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