N-Ethylmaleimide Uncouples the Glucagon Receptor from the Regulatory Component of Adenylyl Cyclase

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ABSTRACT: ^{125}I-Glucagon binding to rat liver plasma membranes was composed of high- and low-affinity components. *N*-Ethylmaleimide (NEM) and several other alkylating agents induced a dose-dependent loss of high-affinity sites. This diminished the apparent affinity of glucagon receptors for hormone without decreasing the binding capacity of membranes. Solubilized hormone–receptor complexes were fractionated as high molecular weight (K_{av} = 0.16) and low molecular weight (K_{av} = 0.46) species by gel filtration chromatography; NEM or guanosine 5′-triphosphate (GTP) diminished the fraction of high molecular weight complexes, suggesting that NEM uncouples glucagon receptor–N-protein complexes. Exposure of intact hepatocytes to the impermeable alkylating reagent *p*-(chloromercuri)benzenesulfonic acid failed to diminish the affinity of glucagon receptors on subsequently isolated plasma membranes, indicating that the thiol that affects receptor affinity is on the cytoplasmic side of the membrane. Hormone binding to plasma membranes was altered by NEM even after receptors were uncoupled from N proteins by GTP. These data suggest that a sensitive thiol group that affects hormone binding resides in the glucagon receptor, which may be a transmembrane protein. Alkylated membranes were fused with wild-type or *cyc*$^-$ S49 lymphoma cells to determine how alkylation affects the various components of the glucagon–adenylyl cyclase system. Stimulation of adenylyl cyclase with fluoride, guanylyl 5′-imidodiphosphate, glucagon, or isoproterenol was observed after fusion of *cyc*$^-$ S49 cells [which lack the stimulatory, guanine nucleotide binding, regulatory protein of adenylyl cyclase (N_s)] with liver membranes alkylated with 1.5 mM NEM. As the NEM concentration increased to 5 or 20 mM, stimulation of adenylyl cyclase by each reagent diminished, suggesting that N proteins were alkylated. Glucagon stimulation of adenylyl cyclase was impaired in fusions of wild-type S49 cells and alkylated liver plasma membranes, suggesting the presence of a thiol in the glucagon receptor that is important for coupling with N_s. These data demonstrate the presence of multiple reactive thiol groups in the glucagon-sensitive adenylyl cyclase system.

Ligand binding to specific receptors on the surface of target cells is the first step in the actions of peptide hormones and neurotransmitters (Kahn, 1976). For example, the binding of a β-adrenergic agonist or glucagon to receptors initiates a sequence of events during which ternary ligand–receptor–guanine nucleotide regulatory protein (N) complexes are formed (Rodbell, 1980). Such complexes play an important role in transmembrane signalling by receptors that activate adenylyl cyclase (Gilman, 1984).

Protein-modifying reagents, such as the alkylating agent *N*-ethylmaleimide (NEM),[1] have been used to probe ligand–receptor and receptor–effector interactions (Moore & Raftery, 1979; Stadel & Lefkowitz, 1979; Heidenreich et al., 1982; Korner et al., 1982; Iyengar & Birnbaumer, 1982; Yamada & Donner, 1985). For example, exposure of membranes to NEM during incubation with a β-adrenergic agonist alkylated a thiol group in the N protein that "locked" agonist–receptor complexes into a high-affinity state that was resistant to dissociation by GTP (Heidenreich et al., 1982; Korner et al., 1982).

This study demonstrates that *N*-ethylmaleimide alkylates multiple reactive sulfhydryl groups in the glucagon-sensitive adenylyl cyclase system. We have confirmed the presence of an alkylatable moiety in N_s (Korner et al., 1982) which eliminates its ability to stimulate adenylyl cyclase. Alkylation of another thiol that is probably in the glucagon receptor uncouples receptors from N proteins. These observations suggest that alkylating agents may be used to characterize and distinguish the interactions of various ligand–receptor complexes with N proteins.

Experimental Procedures

Materials. Lubrol PX, *N*-ethylmaleimide, DTNB, and PCMBS were from Sigma Chemical Co. Ultrogel AcA 34 was from LKB Instruments, Inc. Gpp(NH)p was from Pharmacia P-L Biochemicals, and Na^{125}I was from Amersham Corp. Wild-type and *cyc*$^-$ S49 cells (from Dr. John Bilezekian, Columbia University) were cultured as described by Ross et al. (1977). The sources of other materials were previously described (Corin et al., 1982).

Methods. Glucagon was iodinated according to Greenwood et al. (1963) as modified by Lesniak et al. (1973). ^{125}I-Glucagon was separated from ^{125}I$^-$ by selective desorption from a microfine silicate QUSO 32 (Corin et al., 1982). Partially purified liver plasma membranes were prepared from 150–

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; cAMP, adenosine cyclic 3′,5′-phosphate; cGMP, guanosine cyclic 3′,5′-phosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; HBSS, Hank's balanced salt solution; Gpp(NH)p, guanylyl 5′-imidodiphosphate; GTP, guanosine 5′-triphosphate; NEM, *N*-ethylmaleimide; N_s, stimulatory, guanine nucleotide binding, regulatory protein of adenylyl cyclase; PCMBS, *p*-(chloromercuri)benzenesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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200-g male Sprague-Dawley rats by the Neville procedure (1968) as described by Pohl et al. (1971). The concentration of protein in membranes was measured by the method of Lowry et al. (1951) or by the fluorescamine method (Böhlen et al., 1973) using bovine serum albumin (fraction V) as a standard. Hepatocytes were isolated as described by Berry and Friend (1969) and subsequently maintained in Waymouth's supplemented medium (Terris & Steiner, 1975).

Binding of ^{125}I-glucagon to plasma membranes was performed at 23 °C in the presence of bovine serum albumin (0.5%) in Hank's balanced salt solution (HBSS), which contains 0.9 mM Mg^{2+}, pH 7.4. Triplicate aliquots (100 μL) were assayed for hormone binding by microcentrifugation (1 min, 10000*g*) through 200 μL of 5% sucrose (0 °C) (Corin et al., 1982). The membrane pellet was washed once with 200 μL of ice-cold 5% sucrose and centrifuged for 30 s. The tip of each microcentrifuge tube was cut off for measurement of ^{125}I. Radioactive uptake by membranes in the presence of ^{125}I-glucagon is defined as total hormone binding. Nonspecific binding is defined as the uptake of ^{125}I-glucagon in the presence of unlabeled glucagon (4–26 μM). Specific binding is the difference between total and nonspecific binding. Binding isotherms were analyzed by nonlinear regression as previously described (Donner et al., 1980). Each experiment has been repeated at least twice with results similar to those shown.

The dissociation of ^{125}I-glucagon from liver plasma membranes was measured by vacuum filtration through cellulose acetate filters [Millipore type EG (0.2 μm)]. The filters were soaked in 5% bovine serum albumin for 1 h at 23 °C, mounted on a filtration manifold, and washed with 10 mL of dissociation medium (0.1% bovine serum albumin in HBSS, pH 7.4, 23 °C) immediately prior to use. Dissociation of bound ^{125}I-glucagon was initiated by 100–200-fold dilution of incubates into hormone-free medium. Binding assayed by filtration of six 10-mL aliquots immediately after dilution was defined as the zero time point. At various times thereafter, triplicate aliquots were removed from the incubates for assay of binding. To characterize the effect of GTP on the dissociation of bound ^{125}I-glucagon, a freshly prepared stock solution of this reagent was added to the dissociation medium immediately after assay of binding at zero time. The data are presented as a fraction of ^{125}I-glucagon remaining specifically bound at any time relative to specific binding at the zero time point.

The properties of solubilized hormone–receptor complexes were characterized by gel filtration chromatography. Membranes were incubated with ^{125}I-glucagon (30 min, 23 °C), centrifuged (80000*g*, 10 min), resuspended in a solubilization buffer [25 mM Tris (pH 7.4), 1 mM EDTA, 1% Lubrol PX, 4 °C, and 4.7 mg of protein/mL], and incubated on ice for 30 min. Insoluble material was then removed by centrifugation (185000*g*, 30 min). An aliquot of the supernatant (0.8 mL, about 70000 cpm) was applied to an Ultrogel AcA 34 column (0.9 × 100 cm) equilibrated with elution buffer [25 mM Tris (pH 7.4), 1 mM EDTA, and 0.01% Lubrol PX, 4 °C] and eluted in 1-mL fractions at 12 mL/h. The void (fraction 39) and salt (fraction 94) volumes of the column were determined by elution of blue dextran and reduced DTNB, respectively. Degradation products of ^{125}I-glucagon (assayed as trichloroacetic acid soluble radioactive label) eluted near the salt volume (K_{av} = 0.87–1.06), and intact hormone eluted slightly after the salt volume of the column (K_{av} = 1.09) (not shown), suggesting that ^{125}I-glucagon interacts with the column.

NEM-treated liver plasma membranes were fused with S49 lymphoma cells as described by Steiner and Schramm (1985). Membranes were incubated with *N*-ethylmaleimide (1.5, 5, or 20 mM) at 0 or 23 °C for 30 min. The incubation was terminated by addition of 10 mM Tris, pH 7.4, containing 3.8 mM 2-mercaptoethanol (final concentration 2.5 mM) and centrifugation (80000*g*, 10 min). After an additional wash with the same buffer (1 mL, 0 °C), the alkylated membranes were treated with phospholipids (Sigma crude lecithin, 0.32 mg/mg of membrane protein) as described by Neufeld et al. (1980). The fusion procedure was initiated by centrifuging wild-type or *cyc*$^-$ S49 cells (3×10^7) over a pellet of alkylated membranes (370 μg of protein). The membrane/cell pellet was incubated for 2 min at 30 °C, resuspended in fusion medium [52% w/w poly(ethylene glycol) 6000 in HBSS without calcium or magnesium containing 2.5 mM NaOH, 20 mM Tris, pH 7.5, 2 mM ATP, 5 mM $MgCl_2$, 0.1 mM EDTA, and 5 μg of leupeptin/mL], and incubated at 30 °C for 2 min. The suspension was diluted with 1 mL of fusion medium that did not contain poly(ethylene glycol) or NaOH and after 2 min was diluted with 9 additional mL of this medium and centrifuged (48000*g*, 20 min). With the use of a 22-gauge needle attached to a hypodermic syringe, the pellet was resuspended into 25 mM Tris buffer and 20 μg of leupeptin/mL, pH 7.5 at 0 °C, to hypotonically lyse the cells. The fused membranes were centrifuged (5 min, Beckman Microfuge B) and resuspended into 0.6 mL of HBSS without calcium or magnesium at 0 °C.

Adenylyl cyclase was assayed by the procedure of Salomon et al. (1974) in 40 mM Tris, pH 7.6 (30 °C), 0.1% bovine serum albumin, 5 mM $MgCl_2$, 1 mM EDTA, 2 mM isobutylmethylxanthine, 20 mM phosphocreatine, 0.25 mg/mL (40–50 units/mL) creatine phosphokinase, 0.1 mM ATP, 25 μCi/mL [α-^{32}P]ATP, and 1.25 μCi/mL [2,8-^{3}H]cAMP. The enzyme in one-fifteenth of the liver plasma membrane–S49 cell fusion was assayed in duplicate for 10 min at 30 °C. Formation of cAMP was halted by boiling for 1 min. Activity was measured in the presence of water (basal), fluoride (10 mM), Gpp(NH)p (10 μM), glucagon (1 μM) + GTP (0.2 μM), or (−)-isoproterenol (10 μM) + GTP (0.2 μM). The GTP concentration used to measure glucagon or isoproterenol stimulation of adenylyl cyclase did not significantly increase basal activity.

RESULTS

Exposure of membranes to *N*-ethylmaleimide "locked" bound β-adrenergic agonists into receptor complexes that were insensitive to the dissociation promoting effects of guanyl nucleotides (Heidenreich et al., 1982; Korner et al., 1982). Liver plasma membranes were therefore alkylated to determine if NEM would produce a similar effect on the dissociation of ^{125}I-glucagon (Figure 1, panel A). Control membranes retained 80% of bound ^{125}I-glucagon after 60 min of dissociation. Exposure to *N*-ethylmaleimide (20 mM) during association, or to GTP during dissociation, increased the fraction of hormone that dissociated from membranes such that only 25% of the ^{125}I-glucagon remained bound after 60 min. GTP did not further augment ^{125}I-glucagon dissociation from alkylated membranes.

The results summarized above show that the presence of *N*-ethylmaleimide during an association reaction between ^{125}I-glucagon and liver membranes increases the subsequent release of bound hormone during dissociation. To determine if a similar phenomenon would be observed if hormone binding were allowed to proceed prior to alkylation, liver membranes were incubated with ^{125}I-glucagon for 55 min before the addition of *N*-ethylmaleimide during the final 5 min of association. In this case, results identical with those in which NEM was present throughout association were obtained (Figure 1,

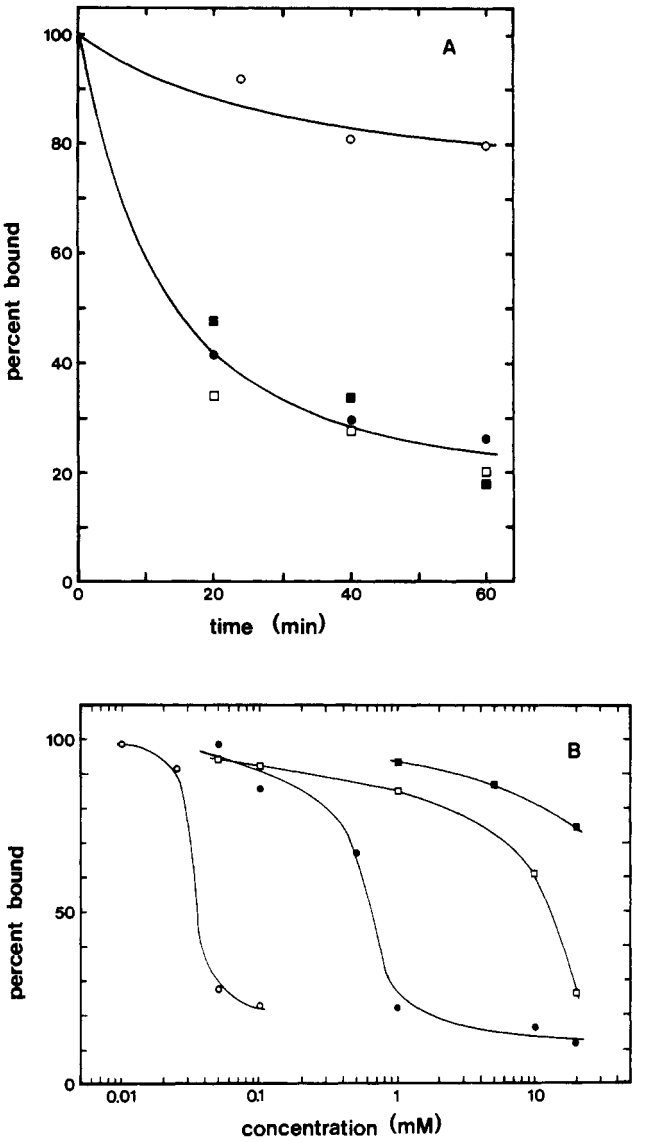

FIGURE 1: Dissociation of ^{125}I-glucagon from liver membranes. (A) Liver membranes (0.3 mg of protein/mL) were incubated with ^{125}I-glucagon (0.9 nM) at 23 °C in the absence (○, ●) or presence (□, ■) of 20 mM *N*-ethylmaleimide for 60 min prior to 200-fold dilution into hormone-free medium. The fraction of hormone remaining bound at any time, relative to binding immediately after dilution, was determined by filtration in the absence of (○, □) or presence (●, ■) of GTP (1.7 μM). (B) Liver membranes (0.3 mg of protein/mL) were incubated with ^{125}I-glucagon (3.1 nM) for 60 min at 23 °C in the absence or presence of various concentrations of PCMBS (○), NEM (●), DTNB (□), or iodoacetamide (■). Bound hormone was determined by filtration immediately after 100-fold dilution and after 60 min of dissociation. The fraction of hormone remaining bound to membranes exposed to alkylating was expressed as a fraction of ^{125}I-glucagon remaining bound to control membranes after dissociation.

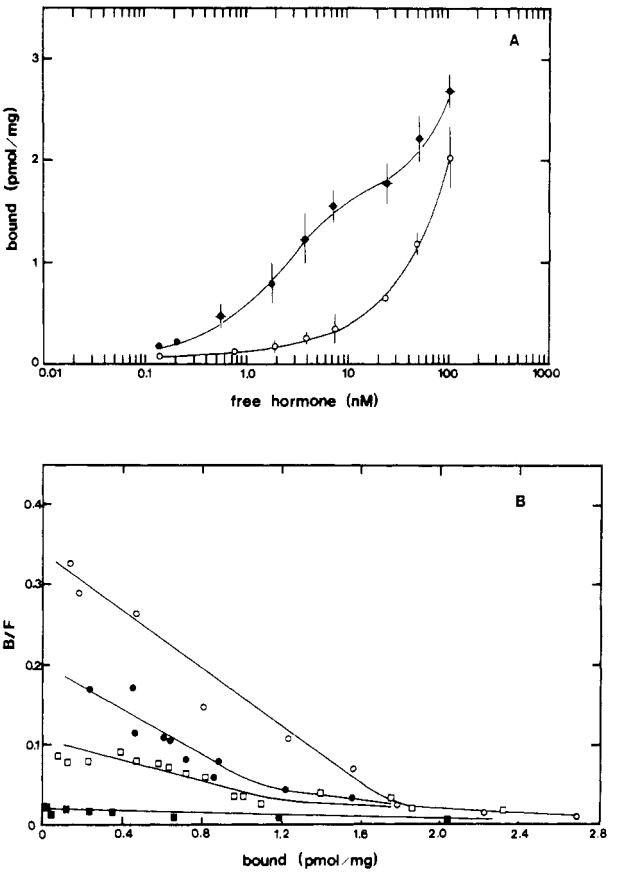

FIGURE 2: Incubation of liver membranes with various concentrations of ^{125}I-glucagon and *N*-ethylmaleimide. (A) Liver membranes (0.31 mg of protein/mL) were incubated for 60 min at 23 °C with various concentrations of ^{125}I-glucagon in the absence (●) or presence (○) of 20 mM *N*-ethylmaleimide. Specific binding was determined by microcentrifugation and is plotted against the logarithm of the free hormone concentration. Each data point represents the mean (±SEM) of triplicate experiments. (B) Liver membranes (0.33 mg of protein/mL) were incubated for 60 min at 23 °C with various concentrations of ^{125}I-glucagon and no additions (control) (○), 0.5 mM NEM (●), 1.5 mM NEM (□), or 20 mM NEM (■). Specific binding was determined and is plotted according to Scatchard (1949). Error bars were omitted for clarity.

panel A). Rapid ^{125}I-glucagon release also occurred if membranes were pretreated with *N*-ethylmaleimide or were exposed to NEM only after incubation with hormone and subsequent dilution to initiate dissociation (not shown).

The ability of *N*-ethylmaleimide and other alkylating agents to enhance ^{125}I-glucagon dissociation was examined (Figure 1, panel B). Membranes were incubated with hormone and various concentrations of an alkylating agent for 60 min prior to dilution into hormone-free medium. After 60 min of dissociation, the amount of hormone retained by alkylated membranes was determined and normalized to the fraction of ^{125}I-glucagon retained by control membranes. The concentration of alkylating agent at which half of the hormone retention of control membranes was observed (EC_{50}) was approximately 0.035, 0.65, and 13 mM for PCMBS, NEM, and DTNB, respectively. Iodoacetamide was less effective than DTNB at promoting ^{125}I-glucagon dissociation.

One explanation for the enhanced dissociation is that alkylation diminishes the affinity of receptors for ^{125}I-glucagon. To test this, membranes were incubated with various concentrations of ^{125}I-glucagon in the absence or presence of 20 mM *N*-ethylmaleimide (Figure 2, panel A). Hormone binding sites of high and low affinity were observed in control membranes. Computer analysis estimated that the higher affinity site bound approximately 2 pmol of ^{125}I-glucagon/mg of membrane protein with a dissociation constant of about 1.8 nM. The lower affinity site was not saturated in the presence of 100 nM hormone. Only lower affinity binding was evident in membranes incubated with NEM. The integrity of unbound hormone was measured by the ability of trichloroacetic acid to precipitate intact, but not degraded, glucagon (Eisentraut et al., 1968). In membranes incubated with *N*-ethylmaleimide, less hormone was degraded than in control membranes, indicating that proteolysis of ^{125}I-glucagon did not account for the diminished affinity of alkylated membranes (not shown).

The loss of high-affinity binding sites upon alkylation was investigated in membranes that were exposed to several con-

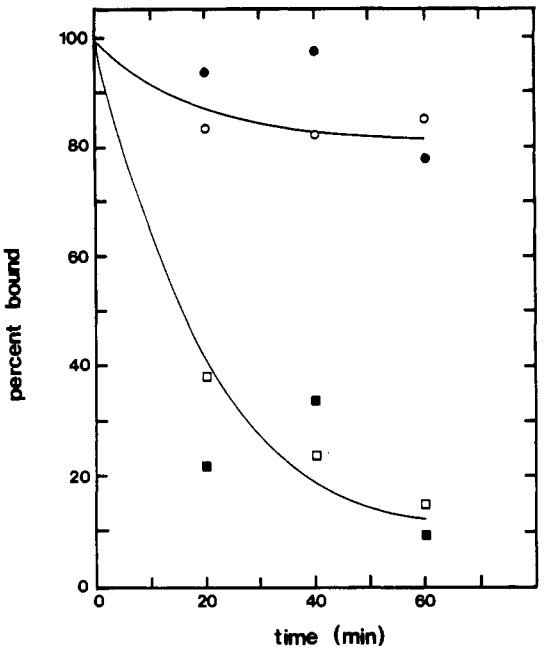

FIGURE 3: Dissociation of ^{125}I-glucagon from plasma membranes isolated from hepatocytes exposed to PCMBS. Isolated hepatocytes were maintained in Waymouth's medium at 37 °C for 30 min and then transferred to HBSS at 23 °C. The intact cells were incubated with HBSS [control (○, □)] or PCMBS [50 μM (●, ■)] for 25 min, centrifuged, and washed twice with 0.25 M sucrose (0 °C). The cells were homogenized in 0.25 M sucrose with a Potter–Elvehjem homogenizer (6 strokes), and the homogenate was centrifuged (3600*g*, 10 min). Partially purified plasma membranes were isolated from the pellet (Pohl et al., 1971). Plasma membranes (0.33 mg of protein/mL) isolated from control or PCMBS-treated hepatocytes were incubated with ^{125}I-glucagon (0.9 nM) for 60 min at 23 °C and then diluted 200-fold into hormone-free medium. The fraction of hormone remaining bound at any time, relative to binding immediately after dilution, was determined by filtration in the absence (○, ●) or presence (□, ■) of GTP (1.9 μM).

centrations of *N*-ethylmaleimide (0, 0.5, 1.5, or 20 mM) (Figure 2, panel B). Binding sites of high and low affinity were evident in control membranes and membranes exposed to 0.5 or 1.5 mM NEM, when ^{125}I-glucagon binding was plotted according to Scatchard (1949). Computer analysis estimated that the higher affinity site bound approximately 1.6 and 1.1 pmol of ^{125}I-glucagon/mg of membrane protein with dissociation constants of about 3 nM in the presence of 0.5 and 1.5 nM NEM, respectively. Higher affinity sites were not observed in membranes incubated with ^{125}I-glucagon in the presence of 20 mM NEM. Although lower affinity sites were not saturated in these experiments, extrapolation of the Scatchard plots suggested that there was no change of total binding capacity (about 4.8 pmol/mg of membrane protein) as the number of high-affinity sites diminished in the presence of increasing concentrations of *N*-ethylmaleimide.

To determine if the alkylation site(s) that regulate(s) glucagon receptor affinity is (are) located on the external surface of the cell membrane, intact hepatocytes were alkylated with PCMBS. ^{125}I-Glucagon dissociation from plasma membranes isolated from control or PCMBS-pretreated hepatocytes was then compared (Figure 3). Hormone dissociated slowly from control and alkylated membranes in the absence of GTP, and approximately 82% of bound ^{125}I-glucagon was retained after 60 min. GTP enhanced hormone dissociation from treated and control membranes, and about 15% of the ^{125}I-glucagon remained bound after 60 min.

Since receptor affinity for glucagon is affected by interaction with N proteins, it is possible that alkylating agents might

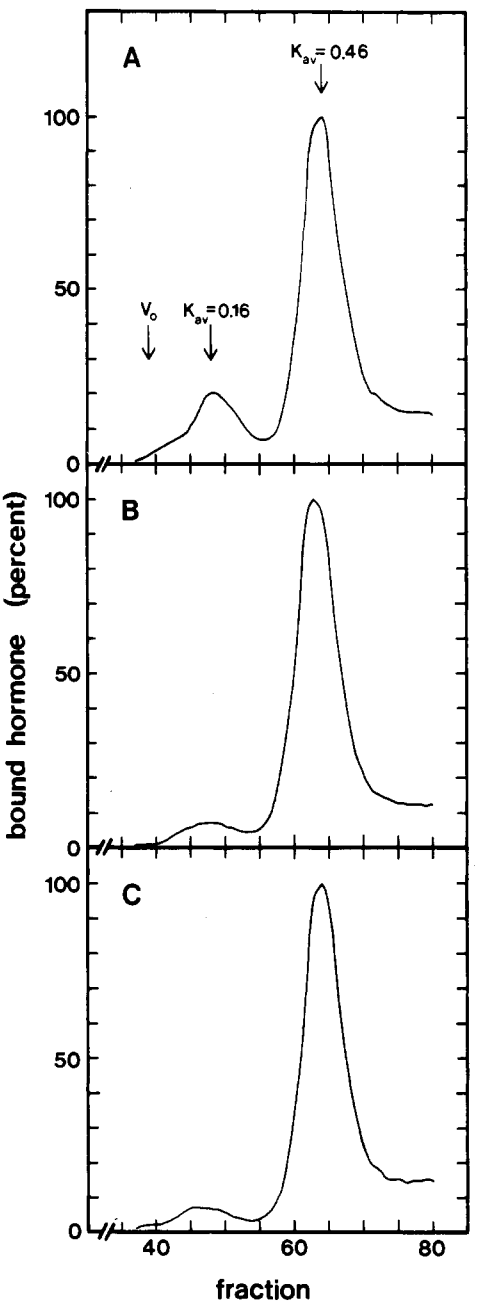

FIGURE 4: Fractionation of solubilized glucagon receptors on Ultrogel AcA 34. Membranes (2.3 mg of protein/mL) were incubated for 30 min at 23 °C with ^{125}I-glucagon (11 nM) and HBSS (panel A), 20 mM NEM (panel B), or 1.7 μM GTP (panel C). The membranes were collected by centrifugation, solubilized, and fractionated on Ultrogel AcA 34 as described under Methods. The sample incubated with GTP was supplemented with fresh nucleotide (100 μM) after solubilization and fractionated in the presence of 10 μM GTP (panel C). The eluted radioactivity is expressed as a fraction of the maximal radioactivity of the second peak (K_{av} = 0.46).

interfere with the formation of glucagon receptor–N-protein complexes. To directly test for such an effect, hormone–receptor complexes were solubilized from control or alkylated membranes and characterized on a gel filtration column. ^{125}I-Glucagon–receptor complexes from control membranes eluted from an Ultrogel AcA 34 column in two peaks of K_{av} 0.16 and 0.46 (Figure 4, panel A). Almost all hormone in high molecular weight complexes (K_{av} = 0.16) was specifically bound. Only 80% of the hormone in lower molecular weight complexes (K_{av} = 0.46) was specifically bound (Lipson et al., 1986) because lipid-detergent micelles also elute in this volume (Welton et al., 1977). The proportion of high to low affinity

and specific to nonspecific binding varies with hormone concentration, making quantitative comparison of gel filtration and equilibrium binding data difficult. However, the ratio of high to low molecular weight complexes (Figure 4, panel A) and high- to low-affinity binding sites (Figure 2, panel B) is qualitatively similar. Exposure of membranes to *N*-ethylmaleimide (Figure 4, panel B) or GTP (panel C) diminished the fraction of ^{125}I-glucagon that eluted in high molecular weight complexes.

The previous data suggest that alkylation may uncouple glucagon receptors from N proteins. To identify whether there is an alkylatable moiety in the glucagon receptor, membranes were exposed to *N*-ethylmaleimide in the presence of GTP. This experiment was based on the premise that if alkylation sites are only in N proteins, hormone association with control membranes in the presence of GTP would be similar to that with alkylated membranes. An alkylated site in the receptor would be suggested by dissimilar binding. Less hormone bound to alkylated than to control membranes throughout an association reaction. The presence of GTP during the association did not affect ^{125}I-glucagon binding to control or NEM-pretreated membranes (Figure 5, panel A). This observation is consistent with those of Rodbell et al. (1971), who have shown that GTP does not diminish the amount of glucagon bound at steady state during an association reaction in the absence of EDTA. The effects of GTP on hormone binding appear to depend on the Mg^{2+} concentration (unpublished results). Similar results were observed, however, if membranes were pretreated with *N*-ethylmaleimide in the presence of GTP and 20 mM Mg^{2+} or in the absence of GTP (not shown). In a related experiment, plasma membranes pretreated with NEM in the presence of GTP were washed free of these reagents and incubated in HBSS for 3 h at 23 °C prior to addition of ^{125}I-glucagon. Results similar to those above were observed, indicating that alkylation is irreversible and does not occur at a site protected by GTP (not shown). Control experiments (showing enhanced dissociation of bound hormone and diminished affinity of receptors for ^{125}I-glucagon in saturation binding assays) confirmed that GTP had uncoupled receptors from N proteins in the experiments described above (not shown).

Somewhat different results were observed if *N*-ethylmaleimide and ^{125}I-glucagon were coincubated with plasma membranes (Figure 5, panel B). There was no difference in binding to control or alkylated membranes during the first 10 min of association. Thereafter, binding to alkylated membranes diminished with time whereas that of control membranes did not. As in the previous experiment, GTP did not alter binding to control or alkylated membranes. These data may suggest that the reactivity of thiol groups varies with time and may be affected by hormone binding.

The catalytic component of adenylyl cyclase is inactivated by low concentrations of alkylating agents (Stadel & Lefkowitz, 1979; Lin et al., 1980). Therefore, in order to characterize how NEM affects interactions between the various components of the adenylyl cyclase system, receptors and N proteins from alkylated liver plasma membranes must be transferred to an unmodified membrane. Wild-type S49 lymphoma cells contain a catecholamine-stimulatable adenylyl cyclase system and lack glucagon receptors (Table I). The *cyc*$^-$ mutant of S49 cells does not express N_s proteins which are essential for hormonal stimulation of the enzyme. Thus, fusion of alkylated membranes with wild-type cells allows the examination of glucagon receptor coupling with unmodified N proteins while fusion with *cyc*$^-$ cells defines the competence

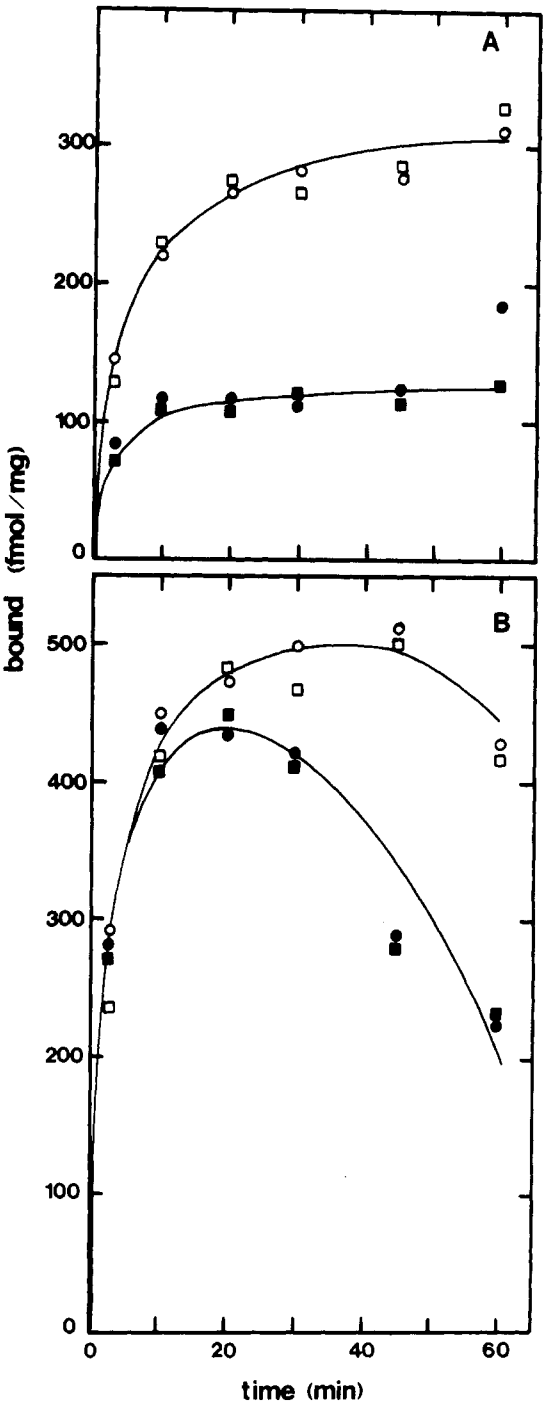

FIGURE 5: Association of ^{125}I-glucagon with liver membranes. (A) Plasma membranes (2.8 mg of protein/mL) were incubated with GTP (46 μM) for 60 min prior to addition of HBSS [control (○, □)] or *N*-ethylmaleimide [1.5 mM (●, ■)]. After 30 min, the membranes were centrifuged (80000*g*, 10 min), washed (5 mL of HBSS, 0 °C), and resuspended in HBSS. Control (○, □) and NEM-pretreated (●, ■) membranes (0.31 mg of protein/mL) were then incubated with ^{125}I-glucagon (0.9 nM) at 23 °C in the absence (○, ●) or presence (□, ■) of GTP (9.2 μM) for various times, and specific binding was measured. (B) Plasma membranes (0.31 mg of protein/mL) were incubated with ^{125}I-glucagon (0.7 nM) at 23 °C in the absence (○, □) or presence of NEM [1.5 mM (●, ■)] and in the absence of (○, ●) or presence of GTP [9.2 μM (□, ■)]. Specific binding was assayed after various times of incubation.

of alkylated N proteins to interact with receptors and adenylyl cyclase.

To optimize the assay of adenylyl cyclase after fusion of membranes with S49 cells, liver plasma membranes were alkylated with various concentrations of NEM at 0 °C and

Table I: Adenylyl Cyclase Specific Activity[a] of *N*-Ethylmaleimide-Pretreated Liver Plasma Membranes and S49 Lymphoma Cells

	basal	fluoride	Gpp(NH)p	glucagon	isoproterenol
pm	20.7 ± 1.3	192.3 ± 24.5	68.8 ± 0.8	70.3 ± 21.9	27.7 ± 4.2
pm_{PL}	3.2 ± 3.0	36.4 ± 13.4	19.6 ± 4.5	9.1 ± 1.7	4.8 ± 0.3
$pm^*_{1.5}$	0.23 ± 0.11	2.44 ± 0.01	1.75 ± 0.19	1.47 ± 0.94	0.36 ± 0.29
pm^*_5	0.85 ± 0.10	0.67 ± 0.85	0.05 ± 0.08	1.46 ± 0.33	0.33 ± 0.44
pm^*_{20}	0.19 ± 0.19	0.81 ± 0.87	0	0.17 ± 0.25	0.47 ± 0.38
cyc⁻	0.28 ± 0.10	0.10 ± 0.14	0.17 ± 0.04	0.18 ± 0.06	0.09 ± 0.12
wt	5.2 ± 0.5	30.6 ± 2.6	7.2 ± 0.6	5.4 ± 0.1	28.4 ± 0.9

[a] The specific activity of adenylyl cyclase ± SEM, determined from duplicate assays, is presented in picomoles per minute per milligram. Abbreviations: wt, wild-type S49 lymphoma cells; *cyc*⁻, *cyc*⁻ mutant of S49 lymphoma cells; pm, liver plasma membranes; pm^*_x, liver plasma membranes pretreated with *x* mM *N*-ethylmaleimide; pm_{PL}, control liver plasma membranes treated with phospholipids.

Table II: Fusion of *N*-Ethylmaleimide-Pretreated Liver Plasma Membranes with S49 Lymphoma Cells and Assay of Adenylyl Cyclase[a]

pm treatment			assay conditions					
NEM (mM)	temp (°C)	cell	basal	fluoride	Gpp(NH)p	GTP	glucagon	isoproterenol
0		*cyc*⁻	1.45 ± 1.48	11.25 ± 1.67	6.69 ± 0.46	1.28 ± 0.50	5.94 ± 2.20	1.62 ± 1.05
1.5	0	*cyc*⁻	0.27 ± 0.17	7.89 ± 4.11	4.47 ± 1.76	0.37 ± 0.06	1.26 ± 0.81	1.72 ± 1.12
5	0	*cyc*⁻	0.31 ± 0.09	2.08 ± 0.50	1.15 ± 0.18	0.53 ± 0.22	0.61 ± 0.19	0.50 ± 0.06
20	0	*cyc*⁻	0.43 ± 0.04	0.86 ± 0.06	0.95 ± 0.34	0.36 ± 0.16	0.27 ± 0.12	0.44 ± 0.01
0		wt	3.31 ± 0.21[d]	35.84 ± 3.75	13.21 ± 0.80	3.15 ± 0.85[e]	11.97 ± 1.79[d]	13.07 ± 1.32
1.5	0	wt	4.10 ± 0.16[b]	49.85 ± 3.12[b]	20.51 ± 0.35[b]	4.59 ± 0.21[b]	8.29 ± 0.45[b]	15.08 ± 1.85[b]
5	0	wt	3.39 ± 0.28	30.63 ± 4.49	13.58 ± 3.97	3.85 ± 0.28	5.79 ± 1.31	9.81 ± 0.94
20	0	wt	3.12 ± 1.03	27.44 ± 13.38	11.94 ± 5.59	4.32 ± 1.80	4.76 ± 1.58	10.53 ± 7.01
0		wt	3.31 ± 0.21[d]	35.84 ± 3.75	13.21 ± 0.80	3.15 ± 0.85[e]	11.97 ± 1.79[d]	13.07 ± 1.32
20	23	wt	2.80 ± 0.91[d]	24.65 ± 4.11	10.20 ± 3.57[c]	2.70 ± 0.62[d]	4.43 ± 0.91[e]	11.90 ± 1.39

[a] The specific activity of adenylyl cyclase ± SEM ($n = 2$, unless otherwise indicated) is presented in picomoles per minute per milligram. Abbreviations: *cyc*⁻, *cyc*⁻ mutant of S49 lymphoma cells, wt, wild-type S49 lymphoma cells; pm, plasma membranes; temp, temperature. [b] $n = 1$. [c] $n = 3$. [d] $n = 4$. [e] $n = 5$.

washed with 2-mercaptoethanol as described by Steiner and Schramm (1985). This procedure produced a dose-dependent decrease of receptor affinity that was somewhat smaller than that described above (not shown). Alkylation of liver plasma membranes with 1.5, 5, or 20 mM NEM diminished basal adenylyl cyclase activity to less than 5% of control. However, in order to eliminate stimulation of adenylyl cyclase by fluoride, Gpp(NH)p, or glucagon, it was necessary to alkylate membranes with NEM concentrations greater than 1.5 mM (Table I).

The ability of various reagents to activate adenylyl cyclase was measured after alkylated liver plasma membranes were fused with S49 lymphoma cells (Table II). The specific activity of adenylyl cyclase was enhanced approximately 6-fold by isoproterenol after fusion of *cyc*⁻ cells with membranes alkylated with 1.5 mM NEM (Table II). This demonstrates that liver plasma membrane N proteins had been transferred to the *cyc*⁻ cell adenylyl cyclase since a similar enhancement was not observed in the *cyc*⁻ cells or liver plasma membranes prior to fusion (Table I). Activation of adenylyl cyclase by fluoride, Gpp(NH)p, glucagon, and isoproterenol was diminished after fusion of *cyc*⁻ cells and membranes alkylated with 5 mM NEM. Statistically significant stimulation of the enzyme was not observed after *cyc*⁻ cells were fused with membranes treated with 20 mM NEM. Stimulation of adenylyl cyclase by glucagon also diminished with increasing NEM concentration after alkylated membranes were fused with wild-type cells (Table II). In contrast to the results observed with *cyc*⁻ cells, a small activation of adenylyl cyclase by glucagon remained when wild-type S49 lymphoma cells (Table II) or murine erythroleukemia (F_c) cells (not shown) were fused with plasma membranes alkylated with 20 mM NEM. While it is difficult to assess contributions to adenylyl cyclase activity by unalkylated sites or unfused membranes, the residual hormonal stimulation probably resulted from the presence of a small number of unalkylated glucagon receptors, since some higher affinity binding sites were observed in the membranes pretreated with 20 mM NEM at 23 °C (not shown).

Discussion

^{125}I-Glucagon binding to plasma membranes was composed of high- and low-affinity components. *N*-Ethylmaleimide induced a dose-dependent loss of higher affinity sites that diminished the apparent affinity of receptor for hormone with no decrease of binding capacity (Figure 2). The effect of *N*-ethylmaleimide on equilibrium binding was also observed as enhanced dissociation of bound ^{125}I-glucagon. Several alkylating agents (NEM, DTNB, and PCMBS) produced a similar dose-dependent enhancement of hormone release from plasma membranes (Figure 1). ^{125}I-Glucagon also dissociated rapidly from alkylated membranes that were washed free of *N*-ethylmaleimide prior to incubation with hormone (not shown). Glucagon does not contain thiol groups (cysteine, cystine) or proline (Bromer et al., 1971), which is about 10^6 times less reactive with NEM than cysteine (Abbott & Schachter, 1976), suggesting that the site of alkylation is in a membrane protein and not the hormone. Thus, alkylation of a thiol group in a membrane protein converts the glucagon receptor from a high- to a low-affinity state.

Receptors activate adenylyl cyclase via N proteins (Rodbell, 1980; Gilman, 1984). Receptors that are coupled to N proteins bind hormone with high affinity, whereas uncoupled receptors have a lower affinity for ^{125}I-glucagon. It has been suggested that GTP diminishes the affinity of receptors for hormone (Rodbell et al., 1971) by promoting disaggregation of receptor–N-protein complexes (Rodbell, 1980). Solubilized receptors fractionate in high molecular weight, GTP-sensitive complexes that partially cofractionate with adenylyl cyclase activity, and lower molecular weight complexes that do not (Welton et al., 1977). In this study, GTP diminished the proportion of high molecular weight complexes (Figure 4, panel C) and augmented dissociation of bound hormone (Figure 1, panel A). Alkylation also diminished the proportion of high molecular weight complexes (Figure 4, panel B) and the amount of higher affinity binding (Figure 2) as well as enhancing dissociation (Figure 1). The observations summarized above suggest that GTP and *N*-ethylmaleimide un-

couple high molecular weight, high-affinity glucagon receptor–N-protein complexes.

N proteins and the catalytic component of adenylyl cyclase are thought to be on the inner surface of the cell membrane and inaccessible to exogenous agents in intact cells, whereas the binding domains of receptors are on the outer surface of the plasma membrane. The orientation of the alkylatable group responsible for uncoupling was tested by exposing intact hepatocytes to PCMBS, a membrane-impermeable alkylating agent (VanSteveninck et al., 1965). The observation of slow, GTP-sensitive dissociation of ^{125}I-glucagon from plasma membranes isolated from the alkylated hepatocytes suggests that this (these) site(s) is (are) on the cytoplasmic surface of the membrane. The ability of NEM to alter the association of hormone with uncoupled receptors (Figure 5) indicates the presence of a reactive thiol in the glucagon receptor that affects hormone binding. These observations suggest that the glucagon receptor may be a transmembrane protein.

To determine how alkylation affects the function of the various components of the glucagon–adenylyl cyclase system, liver plasma membranes were alkylated with various concentrations of NEM and fused with S49 lymphoma cells (Table II). The diminished stimulation of adenylyl cyclase by glucagon after membranes alkylated with increasing NEM concentrations were fused with wild-type S49 cells suggests that alkylation of the receptor inhibits its interaction with unmodified N proteins. Thus, this study indicates that there are reactive thiol groups in glucagon receptors that affect coupling with N_s as well as hormone binding.

Glucagon stimulation of the enzyme was observed after plasma membranes alkylated with 1.5 mM NEM were fused with *cyc*$^-$ S49 cells. As the concentration of NEM increased, a parallel loss of responsiveness to agents that act directly on N proteins [fluoride or Gpp(NH)p] and via receptors (glucagon or isoproterenol) was observed (Table II). This indicates a diminished ability of N_s to stimulate adenylyl cyclase and suggests that alkylation of a thiol in the N protein prevents its activation or its interaction with adenylyl cyclase.

The presence of thiols in receptors as well as regulatory proteins is not without precedent. In the retinal rod outer segment photoreceptor system, the guanine nucleotide regulatory protein transducin, which is similar to N proteins that regulate adenylyl cyclase, activates a cGMP phosphodiesterase after stimulation of rhodopsin by a photon (Fung et al., 1981). Alkylation of a thiol in the transducin α subunit prevents interaction of transducin with rhodopsin (Reichert & Hofmann, 1984; Ho & Fung, 1984). This thiol appears to be resistant to alkylation in rhodopsin–transducin complexes formed after stimulation of the system by light (Reichert & Hofmann, 1984). Lability of the sulfhydryl groups in the α subunit is also altered by a guanine nucleotide induced conformational change (Ho & Fung, 1984). Thus, thiol lability and the effect of alkylation are dependent on the interaction of transducin with the species that regulate it (guanine nucleotides and receptors). Reactive thiols were identified in rhodopsin (Reichert & Hofmann, 1984) as well as the α and β subunits of transducin (Ho & Fung, 1984). Thus, multiple thiol groups with variable reactivities can be present in receptors and regulatory proteins.

As observed for rhodopsin–transducin interactions, the effect of alkylation on catecholamine binding is dependent on the conditions of the experiment. Coincubation of membranes with β-adrenergic agonists and *N*-ethylmaleimide resulted in retention of agonist in high-affinity complexes that were insensitive to dissociation by guanine nucleotides (Heidenreich et al., 1982). The formation of such complexes was not observed in the presence of GTP (Vauquelin et al., 1980) or with *cyc*$^-$ S49 cell membranes which lack functional N_s proteins (Vauquelin & Maguire, 1980), suggesting that alkylation of a thiol in the N protein locked receptors into high-affinity ternary complexes (Korner et al., 1982). Preincubation of membranes with an alkylating agent diminished their affinity for β-adrenergic agonists and inhibited formation of guanine nucleotide insensitive agonist–receptor complexes during subsequent coincubation of membranes with NEM and an agonist (Stadel & Lefkowitz, 1979; Andre et al., 1982). However, β-adrenergic agonists that were bound to membranes prior to alkylation remained in high-affinity complexes sensitive to dissociation by GTP (Stadel & Lefkowitz, 1979). These studies suggest that the reactivity of N-protein thiol group(s) may be determined by conformational changes induced by interaction with receptors and guanine nucleotides. It is also possible that a sulfhydryl group in the β-adrenergic receptor affects agonist binding (Stadel & Lefkowitz, 1979; Heidenreich et al., 1982).

In contrast to the effects of alkylation on catecholamine binding, exposure of liver membranes to *N*-ethylmaleimide before (not shown), during (Figures 1 and 2), or after (not shown) association with ^{125}I-glucagon diminished the affinity of the glucagon receptor for hormone. Glucagon and catecholamines also affect cellular sensitivity differently. Glucagon, but not catecholamines, induces heterologous as well as homologous desenstization of adenylyl cyclase (Noda et al., 1984; Rich et al., 1984). Heterologous desensitization results from altered regulation of common N proteins that mediate receptor action (Noda et al., 1984; Rich et al., 1984). This report suggests that alkylation affects the interaction of glucagon and catecholamine receptors with N_s differently. This is an interesting result since it has been generally assumed that receptors that activate adenylyl cyclase contain distinct and specific ligand binding sites but similar sites for coupling to N_s. The distinguishable interactions of various receptors with N proteins may be important to understanding mechanisms that regulate hormonal stimulation of adenylyl cyclase.

In summary, thiol alkylation diminishes the affinity of glucagon receptors for hormone. This occurs by conversion of high-affinity, coupled receptors to low-affinity receptors that are uncoupled from N proteins. Glucagon–adenylyl cyclase systems can be alkylated at a number of sites. One alkylatable group, which uncouples receptors from N proteins, is on the cytoplasmic surface of the membrane and may be in the glucagon receptor. Other groups in N proteins affect stimulation of adenylyl cyclase. The ability of *N*-ethylmaleimide to alter interactions between various receptors and N proteins differently may help to reveal how such interactions occur and are regulated.

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