Isoproterenol stimulates shift of G proteins from plasma membrane to pinocytotic vesicles in rat adipocytes: A possible means of signal dissemination

(cAMP/subcellular fractionation/cholera toxin/pertussis toxin/Western blotting)

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ABSTRACT Guanine nucleotide-binding regulatory proteins (G proteins) are linked to a large number of surface membrane receptors and appear to regulate a variety of effector systems located both in the plasma membrane and in other parts of the cell. The mechanism of the disseminative actions of G proteins remains obscure. During an investigation of the fate of two types of G proteins, Gs and Gq, in rat adipocytes, we unexpectedly found that isoproterenol, which stimulates cAMP levels and lipolysis in these cells, induces parallel increases in both Gs and Gq in a low-density microsomal fraction rich in endosomes and Golgi bodies. Two plasma membrane constitutive enzymes, adenyl cyclase and 5'-nucleotidase, are also elevated in this fraction. NaF and NaN3, metabolic inhibitors, block the redistribution process. The isoproterenol-stimulated shifts are completely reversible after removal of the hormone, indicating a recycling, endocytotic process. The endocytotic process seems to be fluid phase endocytosis, or pinocytosis, since isoproterenol stimulates the uptake of both fluorescent-labeled dextran and horseradish peroxidase into the same vesicles containing Gq. However, the vesicles that accumulate in response to isoproterenol seem heterogenous in properties that may reflect the lipocytic process induced by isoproterenol. It is speculated that the "pinosomes" formed in response to lipocytic hormones may continually produce signals within the cellular interior during their processing and cycling. Hence, signal production in response to hormones need not be confined to the cell membrane; circulating pinosomes may be responsible for some of the disseminative effects of hormones.

GTP-binding proteins share characteristics of binding and degrading GTP to GDP. One group of these proteins is associated with the actions of numerous hormones and neurotransmitters at the plasma membrane. These proteins, Ga proteins, are distinct from other groups because when isolated they are associated with a complex of two other proteins designated β/γ. The heterotrimeric complexes are termed G proteins (see ref. 1 for review). An essential question is how these proteins regulate what seems to be a multitude of functions. A related logistical problem stems from the fact that some of these functions are not confined to the cell membrane where the transduction elements are generally localized. One possibility, embodied in the "pro-grammable messenger theory" (2), is that the Ga proteins dissociate from the β/γ subunits when activated by the synergistic actions of GTP and hormones; the liberated proteins may diffuse throughout the cell and react with effectors both at the cell membrane and at various other compartments in the cell. However, it has not been demonstrated in either cells or with isolated cell membranes that Ga proteins are released in response to the combined actions of GTP and hormones or neurotransmitters.

We have chosen the rat adipocyte to investigate the fate of Ga proteins and β/γ complexes under the influence of hormones. By using cholera toxin (CTX) and pertussis toxin (PTX) labeling and immunoblotting for detection of two types of Ga proteins (αi and αq) and the β subunits, it was unexpectedly found that each of these subunits declined in parallel in subcellular fractions enriched in the plasma membrane after activation of β-adrenergic receptors by isoproterenol. Similarly 5'-nucleotidase and adenyl cyclase, enzymes that are typical plasma membrane (PM) markers, displayed parallel decreases. In all cases, these changes were accompanied by the appearance of the marker enzymes, the α proteins, and the β subunits (presumably as β/γ complexes) in a low-density microsomal (LDM) fraction known to be enriched in endosomes and Golgi membranes. The changes in distribution were energy-dependent and reversible. The present study documents these findings and provides evidence that the observed vesiculation of the plasma membrane in fat cells reflects fluid phase endocytosis, or pinocytosis. Discussed is the possibility that the pleiotropic actions of hormones acting through G proteins need not be confined to the cell membrane. At least in adipocytes, hormones may generate signals continually during the cycling and processing of endocytotic vesicles containing the transduction elements required for signal production; the "pinosomes" may serve as vehicles for conveying information throughout the cellular interior.

MATERIALS AND METHODS

Reagents. [32P]NAD+, [α-32P]ATP, uridine diphosphate galactose, and 5'-[3H]AMP were purchased from New England Nuclear. PTX and CTX were from List Biological Laboratories (Campbell, CA). Collagenase (type 1) was from Worthington. Bovine serum albumin (BSA; type 5, fatty acid free) was from Armour Research Laboratories. Fluorescein isothiocyanate-labeled dextran (FITC-dextran) (M, 70,000) and all other chemicals were either from Sigma or were the highest quality available.

Antiserum. Goat anti-rabbit IgG and rabbit peroxidase-anti-peroxidase were from Organon Teknika-Cappel. Rabbit antiserum AS/6 and RM/1 were generous gifts from Allen Spiegel (National Institutes of Health, Bethesda, MD). Rabbit antiserum U49 was kindly provided by Suzanne Mumby (Department of Pharmacology, Southwestern Medical Center).

Abbreviations: G proteins, guanine nucleotide-binding regulatory proteins; Ga proteins, G proteins involved in signal transduction; BSA, bovine serum albumin; LDM, low-density microsomes; PM, plasma membrane; PTX, pertussis toxin; CTX, cholera toxin; FITC-dextran, fluorescein isothiocyanate-labeled dextran.

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ter, Dallas). Detailed characterizations of AS/6, RM/1, and U49 have been published (3, 4).

Adipocyte Preparation and Subcellular Fractionation. Adipocytes were isolated by the method of Rodbell (5) as modified by Honnor et al. (6), except that minced adipocytes were incubated with collagenase (type 1) in Krebs–Ringer bicarbonate buffer containing 2 mM glucose and 30 mM Hepes (pH 7.4) with 5% (wt/vol) BSA (KRHB5%). Briefly, epididymal fat pads of 150- to 180-g Sprague–Dawley rats of the CD strain (Charles River Breeding Laboratories) were minced with a tissue chopper. Pooled minced fat pads (4 g) were digested in 12 ml of collagenase (0.75 mg/ml) for 45 min in KRHB5% containing 200 nM adenosine at 37°C. After washing the cells three times with KRHB5%, 1 vol of packed cells [final concentration = 10–15% (vol/vol)] was incubated with or without additives in 4 vol of KRHB5% or Krebs–Ringer bicarbonate buffer containing 30 mM Hepes (pH 7.4) with 1% (wt/vol) BSA (KRHBH1%) for various times as indicated in the legends. During this period, adenosine deaminase (1 unit/ml) was added to the medium. Subcellular fractionation was carried out by the method of Kono et al. (7) with slight modification. Cells were washed once with 0.25 M sucrose in 10 mM Tris–HCl, pH 7.4/5 mM NaCl at 17°C and then homogenized at 4°C in 2 vol of buffer A (10 mM Tris–HCl, pH 7.4/5 mM NaCl/1 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride/leupeptin at 50 μg/ml) containing 0.25 M sucrose. The homogenate was kept on ice for 10–15 min to compact the fat and was subsequently centrifuged in a Beckman SS34 rotor at 13,000 rpm (20,000 × g) for 1.5 min. The infranatant was centrifuged for 30 min at 200,000 × g. The resultant supernatant was removed, and the pellet was resuspended in the above homogenizing medium. The thoroughly dispersed suspension was layered on a sucrose gradient (13–45% wt/vol) containing buffer A. After centrifugation in a Beckman SW41 rotor at 35,000 rpm for 40 min, 0.6-ml aliquots were removed from the top fraction (fraction 1) to the bottom fraction. In most experiments, fractions 2–6 were consolidated as the LDM fraction; fractions 8–12 were consolidated as the PM fraction.

ADP-Ribosylation by PTx and CTx. By using 5 μg of membrane protein, PTx-stimulated ADP-ribosylation was done at 4°C for 3 hr, as reported previously (8), with the toxin at 50 μg/ml. ADP-ribosylation by CTx was carried out by the procedure of Ribeiro-Neto et al. (8). For both PTx- and CTx-stimulated ADP-ribosylation, the [32P]NAD+ concentration was 5 μM in the presence of 0.005% Lubrol. The reactions were stopped by addition of Laemmli buffer (9); the samples were boiled for 3 min and applied to 10% acrylamide gels. Subsequent to SDS/polyacrylamide gel electrophoresis, the gels were transblotted to nitrocellulose paper and autoradiographed (6–24 hr exposure for PTx; 12–48 hr for CTx).

Immunodetection. Immunoblots of the transblotted material were prepared as described (10). Only the relevant portions of the autoradiograms or immunoblots are shown in this paper.

Enzyme Assays. Assays for adenyl cyclase activity were carried out in a reaction mixture containing 25 mM Tris–HCl (pH 7.5), 1 mM EDTA, 5 mM MgCl2, 1 mM cAMP, 0.1 mM [α-32P]ATP, creatine phosphokinase at 0.2 mg/ml, and myokinase at 20 μg/ml at 30°C for 10 min. The production of cAMP was measured by the procedure of Salomon et al. (11). 5′-Nucleotidase activity was measured by the method of Avruch et al. (12). Protein concentration was determined by the method of Bradford (13).

**RESULTS**

Effects of Isoproterenol on Distribution of CTx and PTx Substrates Between PM and LDM Fractions. During initial studies, it was found that incubation of rat adipocytes with isoproterenol led to decreased amounts of CTx-labeled material in sucrose gradient fractions enriched in plasma membranes.* The decreases were variable in amount but were particularly evident when adipocytes were incubated with isoproterenol for periods longer than 15 min and in incubation buffers containing 1% (wt/vol) or less BSA (see below). In subsequent studies, we found decreased levels not only of CTx-labeled substrates but also of PTx substrates labeled with [32P]NAD+ in the plasma membrane-enriched fractions, as shown in Fig. 1 A and B. These unexpected findings led to investigations of the subcellular distribution of the CTx- and PTx-labeled substrates. As seen in Fig. 1, LDM, a sucrose gradient fraction enriched in Golgi membranes and endocytic vesicles (7), showed parallel increases in CTx- and PTx-labeled substrates when adipocytes were stimulated with as little as 0.1 μM isoproterenol. The enhanced levels of these substrates found in the LDM fraction proved more sensitive than the PM fraction for assaying the effects of agents that induced these changes. For example, note in Fig. 1 that increases in CTx-labeled proteins were readily detectable with 0.1 μM isoproterenol in the LDM-enriched fractions, whereas in the corresponding PM fractions the decline in CTx-labeled substrates was barely discernible. This sensitivity of the LDM fraction to the effects of isoproterenol was also noted when the BSA concentration in the incubation medium was varied. Shown in Fig. 1C is the importance of albumin and possibly of free fatty acids (see Discussion) in the observed phenomena. Cells incubated in the presence of 1% BSA showed marked isoproterenol-induced decreases and increases, respectively, in the levels of CTx-labeled substrates in the PM and LDM fractions relative to that seen with 5% BSA in the incubation medium.

**Identification by Immunoblotting of G Proteins.** Although ADP-ribosylation of Ga proteins by toxins is a sensitive assay, this procedure has the inherent problem of requiring cofactors that may vary in amounts in the various subcellular fractions. Accordingly, we turned to detection by immuno-

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*Adenosine deaminase in the incubation medium also stimulates lipolysis to about the same extent given by isoproterenol. However, the enzyme does not induce the shifts in distribution of G proteins (unpublished observation).
blotting (Western blots) of the membrane fractions. Fig. 2 shows typical results utilizing antisera that specifically react with \( \alpha_s \), \( \alpha_t \), and \( \beta \) subunits of G proteins. As was observed with the toxin labeling studies, incubation of adipocytes with isoproterenol for 30 min resulted in parallel shifts of all three subunits of G proteins toward the LDM fractions. Note that the \( \alpha_t \) antiserum detected both CTx-labeled bands shown in Fig. 1; both bands in LDM were increased in response to isoproterenol.

The finding that both \( \alpha \) and \( \beta \) subunits are affected in a parallel manner by isoproterenol suggested that intact G proteins are translocated to the LDM fractions in response to isoproterenol. This finding raised the possibility that isoproterenol induces a general shift in distribution of plasma membrane proteins to the LDM. Support for this possibility were findings that 5'-nucleotidase and adenylyl cyclase, both typical plasma membrane proteins, also shifted to lower density fractions after isoproterenol treatment of adipocytes (Fig. 3). The simplest interpretation of these findings is that the hormone induced or enhanced an endocytic process.

**Energy Dependency.** A number of hormones and other agents that bind to cell surface receptors induce cycling of the receptors and their ligands through complex energy-dependent processing of endocytic vesicles, as in the case of insulin receptors and glucose transporters in adipocytes (14, 15). \( \text{Na}_3 \) and \( \text{NaF} \), in combination, reversibly inhibit metabolism and lipolysis in rat adipocytes (16). As shown in Fig. 4, these agents completely blocked the shift in distribution of PTx-labeled substrates (and CTx-labeled substrates in other experiments) induced by isoproterenol. Moreover, such treatment caused effectively all of the labeled material to be confined to the plasma membrane-containing high-density fraction. The same treatment also affected the plasma membrane-enriched fractions in control cells. Treatment with \( \text{NaF} \) and \( \text{Na}_3 \) did not block the ability of isoproterenol to stimulate cAMP production (data not shown).

**Reversibility of Isoproterenol Action.** To test for reversibility, adipocytes were incubated with or without the hormone for 45 min; the cells were then either washed to remove the hormone and/or incubated with the \( \beta \) blocker propranolol to prevent any bound hormone from acting. Adipocytes were incubated for an additional 45 min in the absence or presence of isoproterenol. Fig. 5 shows that, when the effects of the hormone on the shift of CTx substrates to the PM fraction were stopped by the \( \beta \) blocker during the subsequent 45-min incubation, the shifts were no longer apparent (Fig. 5, lanes 2 and 3), indicating that the endocytic process is completely reversible during this period of time.

**Isoproterenol Stimulates Fluid Phase Endocytosis.** Using FITC-dextran as a probe for monitoring the possible effects of isoproterenol on fluid phase endocytosis (17), we have reported elsewhere that the hormone increases by 30% the amount of FITC-dextran encapsulated in vesicles in the LDM fraction (18). This finding indicated that the hormone stimulates fluid phase pinocytosis. However, FITC uptake alone does not provide direct means of identifying the nature of the vesicles present in this fraction. We therefore turned to another technique (19), which involved incubating the cells in medium containing both FITC-dextran and horseradish per-
oxidase followed by incubating the isolated LDM fraction with dianisidine, which is taken up by the vesicles. Subsequent incubation with H2O2 allowed those vesicles containing peroxidase (and FITC-dextran) to oxidize dianisidine; the resultant dense particles are contained only within those vesicles retaining endosomes. These vesicles, being of higher density, could be separated from vesicles not containing peroxidase (and FITC) by fractionation on sucrose gradients, as shown in Fig. 6. It is seen that the separated higher density endosomes contained Go, as a marker for the presence of plasma membrane elements in the FITC-dextran/peroxidase-containing vesicles.

Signal Transduction in the LDM Fraction. To determine whether the various signal transduction components (G proteins and adenyl cyclase) remained operative in the LDM and PM fractions obtained from isoproterenol-treated adipocytes, we measured the response of adenyl cyclase to various activating agents. Shown in Table 1 are comparative adenyl cyclase activities in LDM and PM fractions from adipocytes that had been treated with 10 μM isoproterenol for 30 min. Activities in response to AlF₃ and forskolin were measured in the absence and presence of 1 mM GTP as a means of assessing the functional expression of both Gs and Gi; the latter requires concentrations of GTP in excess of 10 μM with membranes from rat adipocytes (20). Propranolol was added to control samples to block effects of any endogenously bound isoproterenol. Isoproterenol-stimulated cyclase activity in the PM fractions was about 2-fold greater than basal activity, whereas in the LDM fraction hormonal effects were not statistically significant (data not shown). Both AlF₃- and forskolin-stimulated cyclase activities were at least 10-fold greater than the basal activity, indicating that Gs is functional in both LDM and PM. GTP significantly (P < 0.05) inhibited these activities, suggesting that Gs is present and functional in both membrane fractions. Thus, both Gi and Gs retained their functions when shifted to LDM in response to isoproterenol.

**DISCUSSION**

Our initial goal in this study was to investigate whether hormones that act through receptors coupled to G proteins stimulate the dissociation and consequent release of their α subunits to the cytosolic compartments of the adipocyte. Our investigations led instead to the conclusion that isoproterenol causes redistribution of G proteins along with other PM elements (adenyl cyclase and 5’-nucleotidase) to a low-density membrane fraction that is generally rich in endosomes and Golgi bodies (7). The characteristics of the process induced by isoproterenol are indicative of an endocytic

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**Table 1. Adenyl cyclase activity in LDM and PM from adipocytes treated with 10 μM isoproterenol**

<table>
<thead>
<tr>
<th>Addition</th>
<th>LDM (pmol/min per mg)</th>
<th>PM (pmol/min per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>− GTP</td>
<td>+ GTP, 1 mM</td>
</tr>
<tr>
<td>None*</td>
<td>1.9 ± 0.6</td>
<td>−</td>
</tr>
<tr>
<td>Propranolol</td>
<td>2.8 ± 0.5</td>
<td>−</td>
</tr>
<tr>
<td>AlF₃ (0.1 mM)</td>
<td>21.2 ± 1.1</td>
<td>17.2 ± 1.2</td>
</tr>
<tr>
<td>Forskolin</td>
<td>27.1 ± 0.3</td>
<td>22.1 ± 0.3</td>
</tr>
</tbody>
</table>

*All solutions contained 10 nM GTP.
process. The process is temperature-dependent, is reversible, and is effectively abolished by agents (NaF and NaF) that inhibit metabolism and lipolysis in adipocytes (17). In recent studies, we have found that propranolol or dibucaine, agents that disrupt the cytoskeletal network (21) and thus may affect endocytic processing, also inhibit the isoproterenol-induced process (unpublished observations). Direct evidence that the shift in distribution of PM markers is due to an endocytic process came from findings that isoproterenol stimulates the uptake of FITC-dextran, a marker of fluid phase endocytosis or pinocytosis (17), into the LDM fraction of rat adipocytes (18). Here we show that pinocytic vesicles (containing FITC-dextran and horseradish peroxidase) in the LDM fraction have Gα, (and probably other transduction elements) in their membranes.

Our findings confirm the early observation of Cushman (22) that epinephrine stimulates pinocytosis, as monitored by the uptake of gold particles, in rat adipocytes. Correlated with increased pinocytic activity were increased levels of intracellular unesterified fatty acids as reflected by the effects of albumin concentration. Because of its fatty acid binding capacity, serum albumin controls the egress and thus the accumulation of fatty acids in adipocytes; in time the accumulated intracellular fatty acids retard the lipolytic process (23). In our study, the isoproterenol-dependent shift of G proteins to the LDM fraction was time-dependent (minimally 15 min) and was enhanced at decreased albumin concentrations, which again argues for a relationship between pinocytic activity and intracellular levels of fatty acids. On the basis of such correlations, a role for pinocytic activity in the transport of fatty acids in adipocytes has been suggested (24). We have proposed (18) an alternative, larger role for pinocytic activity in adipocytes and possibly many other cell types that dynamically control the input and output of storage material (fat, protein, and carbohydrate). Briefly, the formation of pinosomes may be a means for conveying to and processing information in various compartments in the cell. Thus, for example, hormonally induced signals such as cAMP may be continually generated during the cycling and processing of the pinosomes. Fusion (regulated possibly by cAMP and activated protein kinases) of pinosomes with vesicles containing storage fat and subsequent hydrolysis of fat by lipases present in or transferred to the fused vesicles would give rise to fatty acid-rich vesicles. These vesicles may be destined for fusion with the cell membrane and consequent secretion of fatty acids to the exterior. At limiting albumin concentrations, the fatty acid-rich vesicles accumulate, thus accounting for the increased levels of LDM vesicles enriched in PM markers at lower albumin concentrations. We have found recently that the LDM fraction from isoproterenol-treated adipocytes also contains higher concentrations of triglycerides (unpublished observations). Heterogeneity of the vesicles derived from pinosomes (i.e., vesicles containing PM markers) is further illustrated by the broad distribution of G proteins over the entire range of sucrose concentrations during gradient fractionation (cf. Fig. 2). Such heterogeneity of pinocytic vesicles is commonly observed and is probably the consequence of multiple processing of pinosomes as they progress through the endocytic transport pathway (25).

In order to understand the role of hormones in the pinocytic process, an investigation of the earliest phases of the pinocytic process prior to multistep processing is necessary. Does isoproterenol stimulate the initiation of pinocytosis or does it affect subsequent processing? What factors determine the on/off regulation of adenylyl cyclase in pinosomes: the residency time of the hormone within the vesicles, the turnover of GTP, and destruction and/or cycling of the pinosomes back to the cell membrane? Are the poorly understood antilipolytic actions of insulin related to the hormone’s actions on various endocytic processes including the cycling of glucose transporters and receptors for insulin-like growth factors (26)? Insulin inhibits the stimulatory effect of epinephrine on pinocytosis (22), possibly through its antilipolytic actions, and exerts a small stimulatory effect on basal pinocytosis (27). The possibility that signals may be generated and disseminated continually within the cell during the cycling and processing of endocytic vesicles obviously raises many additional issues concerning the regulation of cellular responses to hormones.