Promiscuous coupling between G protein-coupled receptors and multiple species of heterotrimeric G proteins provides a potential mechanism for expanding the diversity of G protein-coupled receptor signaling. We have examined the mechanism and functional consequences of dual G_{i}/G_{s} protein coupling of the β_{3}-adrenergic receptor (β_{3}AR) in 3T3-F442A adipocytes. The β_{3}AR selective agonist disodium (R,R)-5-[2-[3-(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate (CL316,243) stimulated a dose-dependent increase in cAMP production in adipocyte plasma membrane preparations, and pretreatment of cells with pertussis toxin resulted in a further 2-fold increase in cAMP production by CL316,243. CL316,243 (5 μM) stimulated the incorporation of 8-azido-[32P]GTP into G_{s} (1.68 ± 0.13; n = 4) in adipocyte plasma membranes, directly demonstrating that β_{3}AR stimulation results in G_{s}-GTP exchange. The β_{3}AR-stimulated increase in 8-azido-[32P]GTP labeling of G_{s} was equivalent to that obtained with the A_{1}-adenosine receptor agonist NE-cyclopentyladenosine (1.56 ± 0.07; n = 4), whereas inclusion of unlabeled GTP (100 μM) eliminated all binding. Stimulation of the β_{3}AR in 3T3-F442A adipocytes led to a 2-3-fold activation of mitogen-activated protein (MAP) kinase, as measured by extracellular signal-regulated kinase-1 and -2 (ERK1/2) phosphorylation. Pretreatment of cells with pertussis toxin (PTX) eliminated MAP kinase activation by β_{3}AR, demonstrating that this response required receptor coupling to G_{i}. Expression of the human β_{3}AR in HEK-293 cells reconstituted the PTX-sensitive stimulation of MAP kinase, demonstrating that this phenomenon is not exclusive to adipocytes or to the rodent β_{3}AR. ERK1/2 activation by the β_{3}AR was insensitive to the cAMP-dependent protein kinase inhibitor H-89 but was abolished by genistein and AG1478. These data indicate that constitutive β_{3}AR coupling to G_{i} proteins serves both to restrain G_{s}-mediated activation of adenyl cyclase and to initiate additional signal transduction pathways, including the ERK1/2 MAP kinase cascade.

Long before the discovery of the β_{3}AR and its recognition as a unique, adipocyte-specific receptor controlling lipolysis and thermogenesis, Rodbell and colleagues (1) made the observation that there was an unusual, biphasic stimulation of cAMP production in adipocytes in response to the β_{3}-adrenergic receptor agonist isoproterenol. Depending upon the concentration of GTP in the assay, isoproterenol could either stimulate or inhibit adenyl cyclase activity in adipocyte plasma membranes. Murayama and Uti (2) showed that this inhibitory phase could be relieved by pretreatment of adipocytes with pertussis toxin (PTX). This curious observation lay fallow until studied later in greater detail by Bégin-Heick (3–5). However, it was not until the cloning and characterization of the β_{3}AR gene and the development of selective β_{3}AR agonists (6, 7) that it was postulated that this novel adipocyte-specific β_{3}AR may be responsible for the biphasic adenyl cyclase response in adipocytes (8). We have previously noted that despite the relatively high level of expression of the β_{3}AR in adipocytes, the efficiency of coupling of the β_{3}AR to stimulation of adenyl cyclase is low (9). However, there has been no clear biochemical demonstration of physical coupling of the β_{3}AR to G_{i}, other than comparative functional experiments in the presence or absence of PTX (10), nor has there been any indication of what additional second messenger pathway may be activated as a consequence of this putative coupling of β_{3}AR to G_{i}.

Recently, many G protein-coupled receptors have been shown to mediate cellular growth or differentiation responses through the activation of MAP kinase cascades (11). Receptors signaling via PTX-sensitive G_{i/o} proteins, as well as PTX-insensitive G_{α}_{11} proteins may activate the ERK1/2 MAP kinase cascade through a mechanism involving tyrosine protein phosphorylation and the activation of the low molecular weight G protein p21^{ ras} (12–14). Little is known about the potential role of βARs in the regulation of the MAP kinase pathway. Recently, we have found that in fibroblasts the β_{3}AR mediates Ras-dependent ERK1/2 activation through its ability to couple to a PTX-sensitive G_{i} protein (15). β_{3}AR coupling to G_{i} occurs as a result of PKA-dependent phosphorylation of the receptor, which effectively “switches” receptor coupling from G_{s} to G_{i} proteins. In contrast, β_{3}AR-mediated ERK1/2 activation in S49 lymphoma cells is an entirely G_{i}-dependent process (16). In this system, PKA-mediated phosphorylation of the low molecu-
ular weight GTPase, Rap1, promotes Ras-independent ERK1/2 activation; this process was shown to be independent of β AR interaction with Gαi proteins. Therefore, it is not yet clear whether there is a common mechanism by which β ARs activate MAP kinase.

Here, we demonstrate that stimulation of the β AR in adipocytes induces the direct activation of both Gαi and Gβ. In these cells, Gαi activation results in both the attenuation of β AR-mediated stimulation of adenylyl cyclase and the activation of the ERK1/2 MAP kinase pathway. Unlike the β AR signal in fibroblasts, β AR activation of the ERK1/2 pathway is independent of cAMP and PKA. These data suggest that the promiscuous coupling of the β AR in adipocytes permits the simultaneous transduction of two independent signaling pathways. This property of the β AR may be responsible, in part, for the unique physiological effects of selective β AR agonists in vivo, such as their potency for stimulating lipolysis (17, 18) and their ability to prevent or reverse obesity (6, 19–22).

MATERIALS AND METHODS

Chemicals—CL316,243 was a gift from American Cyanamid Co. (Paris, France). 3AR agonists 6-Cyclopentyladenosine and 3AR antagonist SR59230A were gifts from Capilano (Paris, France). NIH 3T3-2AR cells were washed in 1 mM bovine serum albumin (fraction V), insulin (50 μg/ml), dexamethasone (1 μM), SR59230A (25 μM), SR59230 A or 6-Cyclopentyladenosine was obtained from RBI (Natick, MA). ICI 118551 was purchased from Cambridge Research Biochemicals (Wilmington, DE). Precaution polyacrylamide gels were obtained from Novex (San Diego, CA).

Cell Culture and Transfections—3T3-F442A adipocytes were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, 100 units/ml penicillin, and 10 μg/ml streptomycin (Life Technologies, Inc.) at 37°C in a humidified 5% CO2 atmosphere. Upon reaching 90% confluence, the cells were stimulated to differentiate into adipocytes by culturing in a differentiation media composed of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 10 μg/ml streptomycin, and 5 μg/ml insulin (23). HEK-293 cells were grown in six-well dishes and transfected with 1.2 μg of pBCh3 or pBCh2 DNA (24) by calcium phosphate co-precipitation (25). Ten h later, cells were washed in 1 mM EGTA in phosphate-buffered saline and incubated in growth medium for 24 h. The cells were then serum-starved for 24 h and treated with PTX (100 ng/ml), propanolol (10 μM), SR59230A (25 μM), CL316,243 (10 μM), or isoproterenol (100 μM), and MAP kinase assays were performed as described below. Cells that were pretreated with PTX were incubated with the toxin for 16–20 h at a concentration of 100 ng/ml. Adipocytes—Plasmainduced adipocytes were prepared as described in Materials and Methods. These cells were sonicated for 5 min, and aliquots (30 μg protein/lane) were resolved by SDS-polyacrylamide gel electrophoresis. ERK1/2 phosphorylation was detected by protein immunoblotting using a 1:3000 dilution of rabbit polyclonal phospho-MAP kinase-specific antibody (Upstate Biotechnology, Inc.) to confirm equal amounts of β-adrenergic agonists or growth factors. The serum-free medium consisted of Dulbecco’s modified Eagle’s medium supplemented with 1 g/liter fraction V bovine serum albumin, 10 mM HEPES, pH 7.4, 100 units/ml penicillin, and 100 μg/ml streptomycin. Five min after the addition of β agonists or growth factors, the medium was removed, and the cells were washed once with the preincubation buffer (34). These cells lysates were sonicated for 5 s, and aliquots (30 μg protein/lane) were resolved by SDS-polyacrylamide gel electrophoresis. ERK1/2 phosphorylation was detected by protein immunoblotting using a 1:3000 dilution of rabbit polyclonal phospho-MAP kinase-specific antibody (Upstate Biotechnology, Inc.) and α-Tubulin (Santa Cruz Biotechnology, Inc.) as secondary antibody. Quantitation of ERK1/2 phosphorylation was performed using a Storm PhosphorImager (Molecular Dynamics). After quantitation of ERK1/2 phosphorylation, nitrocellulose membranes were stripped of immunoglobulin and reprobed using rabbit polyclonal anti-ERK 2 IgG (Upstate Biotechnology, Inc.) to confirm equal amounts of ERK 2 protein.

RESULTS AND DISCUSSION

Stimulation of 3T3-F442A adipocyte plasma membranes with the selective β2 AR agonist CL316,243 resulted in a 3–4-fold increase in adenyl cyclase activity (Fig. 1A). Membranes prepared from cells pretreated with PTX in the absence or presence of 3AR agonists were incubated with intact 3T3-F442A adipocytes. For these whole cell assays, growth media was replaced with serum-free medium 3 h prior to stimulation. The serum-free medium consisted of Dulbecco’s modified Eagle’s medium supplemented with 1 g/liter fraction V bovine serum albumin, 10 mM HEPES, pH 7.4, 100 units/ml penicillin, and 100 μg/ml streptomycin. Then cells were incubated with the indicated concentrations of β agonist in fresh serum-free medium for 10 min at 37°C in the presence of 0.25 mM isobutylmethylxanthine. Reactions were stopped by the addition of ice-cold 5% trichloroacetic acid, and particulate material was removed by centrifugation. The cAMP concentrations from both assay methods were determined by radioimmunoassay using a polyclonal antisera to iodinated cAMP (27). cAMP protein concentrations were determined by the method of Bradford (28).

Western blotting was performed with antibodies specific for individual Gα subunits. Adipocyte membranes (30 μg of protein) were solubilized in SDS-polyacrylamide gel electrophoresis sample buffer and resolved on 12% SDS-polyacrylamide gels in a Laemmli system (34). After electrophoresis, the proteins were transferred to nitrocellulose membranes. The membranes were incubated for 16 h at 4°C with rabbit anti-Gα12, anti-Gα13, or anti-Gαo, 1:1000 dilution, followed by alkaline phosphatase-conjugated goat anti-rabbit antiserum (1:10,000 dilution, Amersham Pharmaacia Biotech) as secondary antibody. Immunoreactive bands were visualized by Storm PhosphorImager (Molecular Dynamics). Synthesis and purification of the 8-azido-[32P]GTP was as described (29, 30) with minor modifications (31). For some experiments, 8-azido-[32P]GTP was purchased from Andotek Life Sciences. The photoaffinity labeling procedure of Offermanns (32) was followed with 1% calf serum prepared in the Gα subunits (33). A recombinant G protein standard mix containing Gαo, Gαi3, and Gαs was included as a positive control (gift of Dr. Pat Casey). The proteins were then transferred to nitrocellulose membranes. The membranes were incubated for 16 h at 4°C with rabbit anti-Gα12, anti-Gα13, or anti-Gαo, 1:1000 dilution, followed by alkaline phosphatase-conjugated goat anti-rabbit antiserum (1:10,000 dilution, Amersham Pharmaacia Biotech) as secondary antibody. Immunoreactive bands were visualized by Storm PhosphorImager (Molecular Dynamics).
Two splice variants of Gα, the same repertoire of G proteins as found in rat white adipocytes: (5, 36). As shown in Fig. 2, 3T3-F442A adipocytes express the agonist concentrations. Cyclic AMP production was measured by radioimmunoassay using polyclonal antisera to iodinated cAMP. The data shown are representative of two dose-response experiments. PTX-pretreated adipocytes were prepared and incubated with increasing agonist concentrations. Cyclic AMP production in response to CL316,243. Membranes of untreated or PTX-pretreated membranes. The data shown are the average of four experiments.

Express the mouse β3AR (data not shown) (35). These results suggest that the β3AR is constitutively coupled to both Gs and Gi, because inhibition of Gi function leads to increased tonic production of cAMP.

Rat white adipocytes have been shown to contain the two splice variants of Gαi and the three isoforms of Gαs (αi1, αi2, αi3) (5, 36). As shown in Fig. 2, 3T3-F442A adipocytes express the same repertoire of G proteins as found in rat white adipocytes: two splice variants of Gαia, Gαi3, and Gαia/(Gαi3). Others have previously shown that adipocytes do not contain the PTX-sensitive Gαi protein (36–39). To determine whether the PTX effects on cAMP production resulted from constitutive coupling of the βAR to both Gi and Gs, we used photolabile GTP analog 8-azido-[^32P]GTP to measure βAR agonist-dependent GTP loading of Gαi and Gαs. This method has been used by several investigators to demonstrate physical coupling between G protein-coupled receptors and individual G protein subunits, based upon several well defined criteria for receptor-G protein interaction (40–42). Because the binding of GTP analogs to Go subunits is highly dependent upon free magnesium concentrations (31), we conducted our experiments in adipocyte plasma membranes under separate conditions favorable for 8-azido[^32P]GTP labeling of each Go family. The βAR is coupled to Gαi and activation of adenyl cyclase. Fig. 3A shows that under high magnesium conditions, which favor receptor-stimulated GTP loading of Gαi, CL316,243 could stimulate specific 8-azido[^32P]GTP labeling of Gαi (1.57 ± 0.12; n = 3; p < 0.001). Under low magnesium conditions, which are optimal for determining receptor-stimulated GTP loading to Gi, there was a similar 1.68 ± 0.13-fold increase (n = 4; p < 0.001) in specific 8-azido[^32P]GTP G protein labeling of the 40–42-kDa Gαi species (Fig. 3B). We obtained similar data from 3T3-F442A membranes (not shown). Fig. 3B also shows that the ability of CL316,243 to stimulate Gi-GTP exchange in adipocyte membranes was equivalent to that obtained with the A1-adenosine receptor agonist N^6-cyclopentyladenosine (1.56 ± 0.07; n = 4; p < 0.001). N^6-Cyclopentyladenosine served as a positive control because the A1-adenosine receptor is expressed in both primary adipocytes (43, 44) and differentiated 3T3-F442A adipocytes (45) and couples to all three Gαi species (46, 47).

A number of Gi-coupled receptors have been shown capable of activating MAP kinase (11). Recently, Duaka and colleagues (15) reported that the β3AR could couple to both Gαi and Gαs, with consequent activation of MAP kinase. We therefore determined whether one of the functional consequences of the coupling of the β3AR to Gi in 3T3-F442A adipocytes was the stimulation of MAP kinase. Intact 3T3-F442A adipocytes treated with CL316,243 (5 μM) exhibited approximately a 2.5-fold stimulation of ERK1/2 phosphorylation (Fig. 4). Activation of the endogenous β3AR in 3T3-F442A adipocytes with the β3AR-selective agonist salbutamol also yielded a similar 2-fold stimulation of ERK1/2 phosphorylation. Both βAR responses were less robust than that observed following activation of endogenous receptors for EGF, consistent with previous findings (13). Also shown in Fig. 4, pretreatment of 3T3-F442A adipocytes with PTX completely blocked ERK1/2 phosphorylation in response to either β3AR or βAR stimulation, but it did not affect stimulation by EGF. The addition of propranolol (0.1 μM) eliminated ERK1/2 phosphorylation in response to salbutamol but had no effect on the activation of MAP kinase induced by CL316,243 (not shown), consistent with a CL-mediated effect exclusively through the β3AR (7).

The relative sensitivity of the β3AR to activate MAP kinase versus cAMP accumulation in adipocytes was assessed by treating intact cells with increasing concentrations of CL316,243 (Fig. 5). Because the MAP kinase assays involve the stimulation of intact cells, we included cAMP dose-response data also from intact cells. The EC50 for cAMP production in these experiments was 12 nM, whereas parallel measurements of MAP kinase activation (EC50 = 280 nM) indicated that the PTX-sensitive coupling of the β3AR to MAP kinase activation is less potent under these conditions. Nevertheless, the dose-response curves for both measurements yielded a unit slope, indicative of the high selectivity of CL316,243 for the β3AR (7, 48). Note that the EC50 for cAMP production in these whole cell experiments is significantly less than that found in plasma membranes (Fig. 1). However, it is generally recognized that dose-response curves as well as ligand binding data from intact cells are shifted “to the left,” when compared with data obtained using isolated membrane preparations (49, 50). These comparative studies shown in Fig. 5 suggest that cAMP production is the
Fig. 3. Labeling of Go subunits by 8-azido-[32P]GTP in adipocyte membranes in response to β2AR stimulation. A, 8-azido-[32P]GTP labeling of Goα in response to no stimulation (NS) or labeling with CL316,243 (CL) (5 μM) in rat white adipocyte membranes. B, 8-azido-[32P]GTP labeling of the 40–42-kDa Goα species in response to no stimulation, N8-cyclopentyladenosine (CPA) (50 nM), and CL316,243 (5 μM) in rat white adipocyte membranes. The effect of excess GTP is also shown. Data shown are the average of three experiments (for Goα), or four experiments (for Goi) and are expressed as fold change over nonstimulated control. *, significantly different from unstimulated control, p < 0.001.

Fig. 4. Effect of β-agonists and PTX on ERK1/2 phosphorylation in 3T3-F442A adipocytes. Quantification of ERK1/2 phosphorylation subsequent to agonist stimulation was determined by Western blotting with phosphospecific anti-ERK1/2 rabbit antibodies. Quantitation was performed using a Storm PhosphorImager (Molecular Dynamics). Data shown are the average of four experiments and are expressed as the fold change over nonstimulated control for salbutamol (1 μM), CL316,243 (5 μM), and EGF (10 ng/ml). Pretreatment with PTX was as detailed under “Materials and Methods.” *, significantly different from unstimulated control by one-way analysis of variance, p < 0.001.

Fig. 5. Comparative dose-dependent stimulation of cAMP production (□) and MAP kinase activation (●) in 3T3-F442A adipocytes. Differentiated 3T3-F442A adipocytes were incubated with increasing concentrations of CL316,243 and processed for measurement of cAMP or ERK1/2 phosphorylation as detailed under “Materials and Methods.” The data were analyzed by nonlinear curve-fitting routines (GraphPad Prism) (9). The results shown are from two independent experiments.

Favored pathway in response to activation of the β2AR in adipocytes. It must be remembered, however, that the catalytic activity of the subsequent kinases and their juxtaposition to substrate targets will ultimately determine the relative importance of these two pathways. Future studies will address these issues.

To determine whether the dual Gs/Gi coupling of the β2AR and activation of MAP kinase was an adipocyte-specific event or a unique feature of the rodent β2AR, we attempted to reconstruct these effects by transiently transfecting the human β2AR into HEK-293 cells. As shown in Fig. 4A, transient transfection of the hβ2AR resulted in a low basal level of ERK1/2 phosphorylation (lane 2), and stimulation with the β3AR agonist increased ERK1/2 phosphorylation ~4-fold above basal level (lane 3), as compared with the 7–8-fold activation achieved by EGF (lane 7). There was no effect of the β3AR agonist on mock-transfected cells (lane 1). This activation of MAP kinase by the hβ2AR was completely blocked by the selective β3AR agonist, SR59230A (lane 4) (51). In addition, the nonselective β2AR agonist isoproterenol at a concentration capable of activating the β2AR (100 μM), in the presence of propranolol, also led to an ~4-fold stimulation of ERK1/2 phosphorylation (lane 5). Finally, PTX completely eliminated MAP kinase activation (lane 6), as observed in 3T3-F442A adipocytes (Fig. 4B). Together these data suggest that the dual coupling of the β2AR to Gs/Gi, along with its activation of MAP kinase, is a general property of this receptor.

Mechanistically, β2AR-mediated MAP kinase activation in fibroblasts requires sequential receptor coupling to Goα and Gβγ, because cAMP-dependent, PKA-mediated phosphorylation of the β2AR is a prerequisite for receptor-Gi coupling (15). To compare the role of cAMP and PKA in βAR-mediated MAP kinase activation in adipocytes, we compared the effects of the PKA inhibitor H-89 on β2AR- and β3AR-stimulated MAP kinase activation. As shown in Fig. 6B, H-89 blocked β2AR-stimulated MAP kinase activation (lane 8), consistent with the results of Daaka et al. (15). In contrast, the β3AR signal was insensitive to H-89 (lane 3). Fig. 6B also shows that MAP kinase activation by the β2AR was sensitive to low micromolar concentrations of the tyrosine kinase inhibitor genestein (lane 4) and to the EGFR receptor tyrosinase AB1748 (lane 5). These results indicate that, like other Goα-coupled receptors, the β2AR employs a tyrosine kinase receptor in its mechanism of recruiting MAP kinase (52, 53). In other experiments, treatment with dibutyryl cAMP enhanced β3AR-stimulated ERK1/2 phosphorylation by ~70%, consistent with the model of PKA-dependent β2AR-Gi coupling. However, stimulation of MAP kinase by the β3AR was unaffected by dibutyryl cAMP (data not shown). Together, these results indicate that MAP kinase activation via the β3AR, unlike the β2AR, is insensitive to
modulation of the PKA pathway. Thus \( \beta_3 \text{AR-mediated MAP kinase activation can be distinguished in several ways from the } \beta_2 \text{AR pathway. First, the } \beta_3 \text{AR is not a substrate for PKA (24). Second, the } \beta_3 \text{AR appears to be constitutively coupled to both } G_s \text{ and } G_i \text{. Third, } \beta_3 \text{AR-stimulated activation of cAMP is not required for activation of the MAP kinase cascade in adipocytes. In contrast to the finding of PKA-dependent } \beta_2 \text{AR-mediated MAP kinase activation in S44 lymphoma cells (16), the } \beta_3 \text{AR pathway in adipocytes is completely PTX-sensitive.}

One of the remarkable features of the \( \beta_3 \text{AR is that treatment with } \beta_3 \text{AR-selective agonists in vitro can prevent or reverse obesity due to either congenital or diet-induced etiology (6, 19–22, 54). These agents are also efficacious over prolonged periods of administration (21), which is quite distinct from the rapid desensitization and down-regulation that is characteris-

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development of this project.

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a

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responses.

It will now be important for us to test the consequence of

three experiments. CL, CL316,243; ISO, isoproterenol.

Fig. 6. HEK-293 cells transiently expressing the human \( \beta_3 \text{AR activate MAP kinase. A, HEK-293 cells were tran-
siently transfected with the } h\beta_3 \text{AR as described under "Materials and Methods." Cells were treated as shown, and ERK1/2

phosphorylation was determined. B, HEK-293 cells transiently expressing human \( \beta_3 \text{AR or human } \beta_2 \text{AR were pretreated with

H-89 (20 } \mu \text{M},\text{ genistein (5 } \mu \text{M), or AG1478 (3 } \mu \text{M) for 40 min before agonist stimula-
tion. The results shown represent one of

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