Maximal β3-Adrenergic Regulation of Lipolysis Involves Src and Epidermal Growth Factor Receptor-dependent ERK1/2 Activation*

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Catecholamine-stimulated lipolysis is primarily a β-adrenergic and cAMP-dependent event. In previous studies we established that the β3-adrenergic receptor (β3AR) in adipocytes utilizes a unique mechanism to stimulate extracellular signal-regulated kinases 1 and 2 (ERK) by direct recruitment and activation of Src kinase. Therefore, we investigated the role of the ERK pathway in adipocyte metabolism and found that the β3AR agonist CL316,243 regulates lipolysis through both CAMP-dependent protein kinase (PKA) and ERK. Inhibition of PKA activity completely eliminated lipolysis at low (subnanomolar) CL316,243 concentrations and by 75–80% at higher nanomolar concentrations. The remaining 20–25% of PKA-independent lipolysis, as well as ERK activation, was abolished by inhibiting the activity of either Src (PP2 or small interfering RNA), epidermal growth factor receptor (EGFR with AG1478 or small interfering RNA), or mitogen-activated protein kinase kinase 1 or 2 (MKK1/2 with PD098059). PD098059 inhibited lipolysis by 53% in mice as well. Finally, the effect of estradiol, a reported acute activator of ERK and lipolysis, was also totally prevented by PP2, AG1478, and PD098059. These results suggest that ERK activation by β3AR depends upon Src and epidermal growth factor receptor kinase activities and is responsible for the PKA-independent portion of the lipolytic response. Together these results illustrate the distinct and complementary roles for PKA and ERK in catecholamine-stimulated lipolysis.

When exogenous nutrients are insufficient to meet energy needs, the sympathetic nervous system stimulates the mobilization of free fatty acids from triacylglycerol stored in white adipose tissue through lipolysis (1). Lipolysis proceeds in a stepwise fashion generating diacylglycerol, monoaoylglycerol, and glycerol while releasing three moles of free fatty acids/mole of triacylglycerol. The catalytic steps responsible for lipolysis have been traditionally understood to involve the β-adrenergic activation of cAMP-dependent protein kinase (PKA)2, whose key targets of phosphorylation are hormone-sensitive lipase (HSL) and perilipin A. These events are required together in a coordinated fashion to bring the lipase to the surface of the lipid droplets and are key steps in the lipolytic response to catecholamines (2–8). More recently, the involvement of a novel lipase was predicted when the HSL knock-out mice exhibited a relatively normal triacylglycerol hydrolysis, preferentially accumulating diacylglycerol, and actually a greater deficit in cholesterol ester hydrolysis (9). This revealed that HSL is the rate-limiting step of diacylglycerol, but not triacylglycerol, hydrolysis, because one or more additional lipase(s) could apparently bring about triacylglycerol hydrolysis (9). In that respect a novel hormone-responsive adipose tissue triacylglycerol lipase (also referred to as desnutrin or calcium-independent phospholipase A2, ζ), has been described (10). Targeted disruption of the adipose tissue triacylglycerol lipase gene in mice unequivocally demonstrates its necessity for lipolysis (13). Interestingly, although adipose tissue triacylglycerol lipase is a phosphoprotein, it is not a PKA substrate (10), leaving its mode of activation unclear. Additional triacylglycerol hydrolases have been described in adipocytes, but their contributions to catecholamine-stimulated lipolysis have not yet been assessed (11).

Previously we showed that the β3AR, which is expressed primarily in adipocytes, is coupled to the two heterotrimeric G-proteins, Gα and Gβ (12). This dual coupling permits two parallel signaling pathways to be activated: (i) CAMP generation and PKA activation, and (ii) c-Src recruitment to the receptor and activation of ERK (13). We then proposed that ERK activation might be responsible, at least in part, for the lipolytic response to catecholamines or a β3AR agonist. Subsequently, an involvement of ERK in the lipolytic response was described but not characterized (14). Therefore, we have investigated both the relative contribution of the PKA and ERK signaling pathways in β3AR-stimulated lipolysis in 3T3-L1 adipocytes.

‡This work was supported by National Institutes of Health Grant RO1-DK57698 (to S. C.) and a fellowship from Fonds de la Recherche en Santé du Québec (to J. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: PKA, cAMP-dependent protein kinase; β3AR, β3-adrenergic receptor; CL, (CL316,243) disodium (R,R)-5-[2-[(3-chlorophenyl)-2-hydroxyethyl]-amino]-propyl]-1,3-benzenedi-oxazole-2,2-dicarboxylate; ERK, extracellular signal-regulated kinase; E2, estradiol; HSL, hormone-sensitive lipase; EGFR, epidermal growth factor receptor; siRNA, small interfering RNA; NE, norepinephrine; BSA, bovine serum albumin; PBS, phosphate-buffered saline; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PD, PD098059.
and probed the upstream mechanism of ERK activation. The conclusions derived from these studies show that β₃AR agonists, in addition to their high intrinsic potency toward PKA-mediated lipolysis, activate ERK in a Src- and epidermal growth factor receptor (EGFR)-dependent manner. This ERK activation in the adipocyte serves as a supplemental mechanism to augment and maximize the lipolytic response.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chicken anti-rat HSL antisera were described previously (15). 3T3-L1 cells were obtained from Dr. Howard Green (16). CL316,243 (CL) was a gift from Dr. Elliot Danforth, American Cyanamid Co. (Pearl River, NY). EGFR and Src Smartpool siRNA were from Dharmacon (Chicago, IL). siPORT Lipid was from Ambion (Austin, TX). Anti-p42/44, anti-phospho-p42/44 mitogen-activated protein kinase (MAPK) and anti-Src antibodies were from Cell Signaling Technology (Beverly, MA). Anti-EGFR was from Santa Cruz Biotechnology (Santa Cruz, CA). Kemptide was from Biomol (Plymouth Meeting, PA), dexamethasone, β-estradiol, N-[2-(p-bromocinnamylamino)-ethyl]-5-isouquinoline-sulfonamide (H89), 3-isobutyl-1-methylxanthine, insulin, norepinephrine (NE), prazosin, (+-) propranolol, and yohimbine were from Sigma. Rp-cAMPS was from BioMol, (4-amino-5-(4-chloro- chloroanilino)-6,7-dimethoxyquinazoline (AG1478) were from Calbiochem. Calf serum and fetal bovine serum were from American Cyanamid Co. (Pearl River, NY). EGFR and Src in siPORT lipid. Kemptide (17). In addition, these incubations were done in the presence or absence of H89 (10 μM) or cAMP (5 μM) in order to quantify the PKA-dependent phosphorylation. At the end of the incubation the tubes were placed on ice and the reaction was stopped by the addition of 10 μl of 1% (w/v) BSA, 1 mM ATP (pH 3.0), and 10 μl of 12.5% (w/v) trichloroacetic acid and centrifuged at 12,000 × g for 15 min. Aliquots (20 μl) of the supernatants were removed and applied to phosphocellulose paper (Whatman P-81 paper, VWR). After three washes in 75 mM H₃PO₄ and one wash in acetone, the amount of [γ-³²P]ATP retained was determined by scintillation counting. ERK MAP kinase activation assays were performed as previously detailed (12), or the alkaline phosphatase activity was determined colorimetrically with the Western Blue Stabilized Substrate from Promega (Madison WI).

**Cell Culture and siRNA Treatment**—3T3-L1 cells were maintained at 37 °C in high glucose Dulbecco’s modified Eagle’s medium with 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% calf serum in a 5% CO2 environment. Cells were differentiated 1 day after confluence by replacing calf serum with fetal bovine serum and by adding 0.5 mM 3-isobutyl-1-methylxanthine, 0.4 μg/ml dexamethasone, 5 μg/ml insulin, and 10 μM troglitazone to the medium. After 3 days, cells were maintained for an additional 6–9 days in the same medium without additives, and fresh medium was replenished every 3 days. For the siRNA treatment, cells were differentiated for 6 days and then trypsinized and seeded on dishes containing 25 nM siRNA of EGFR or Src in siPORT lipid.

**Lipolysis**—Differentiated 3T3-L1 adipocytes were washed with PBS and incubated in Kreb’s Ringer buffer containing 2% fatty acid-free BSA at 37 °C for 3 h (for experiments with E₂, cells were incubated overnight). This medium was then replaced with fresh medium containing various concentrations (0, 10–50 μM) of the PKA inhibitors H89 or Rp-cAMPS (0.5 mM) and/or the MEK1/2 inhibitor PD098059 (0, 10–50 μM) for 1 h. After this preincubation, CL or NE (0, 0.1–1000 nM) was added for an additional 1 h. All incubations with NE were preceded by a 2-min preincubation with a mixture of adrenergic receptor (AR) blockers (to limit the action of NE to β₃AR only) consisting of 10 μM prazosin, 10 μM yohimbine, and 0.1 μM propranolol. The glycerol released into the buffer was measured by the GPO-Trinder method (DMA, Inc., Arlington, TX).

**Protein Kinase Activity**—PKA activity was measured in vitro from whole cell extracts prepared from differentiated 3T3-L1 adipocytes that had been pretreated as described above for the lipolysis experiments. After this preincubation, CL or NE (0, 0.1–1000 nM) was added to the cells for 5 min. All incubations with NE were preceded by a 2-min preincubation with the mixture of AR blockers. Cells were washed with PBS, and 200 μl of a lysis buffer (25 mM Tris-HCl, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.5 μM phenylmethylsulfonyl fluoride) was added. The cells were then collected and homogenized by five passages through a 23-gauge needle, centrifuged (12,000 × g, 4 °C) for 10 min, and the infranatant (cytosolic fraction) was collected. A 10-μl aliquot of this extract was incubated for 20 min at 30 °C with 30 μl of a phosphorylation mixture containing 25 mM Tris, pH 7.4, 250 mM sucrose, 50 mM MgSO₄, 5 mM dithiothreitol, 200 μM ATP containing 20 μCi of [γ-³²P]ATP, and 5 μg of Kemptide (17). In addition, these incubations were done in the presence or absence of H89 (10 μM) or cAMP (5 μM) to determine the presence or absence of H89 (10 μM) or cAMP (5 μM) to quantitate the PKA-dependent phosphorylation. The at the end of the incubation the tubes were placed on ice and the reaction was stopped by the addition of 10 μl of 1% (w/v) BSA, 1 mM ATP (pH 3.0), and 10 μl of 12.5% (w/v) trichloroacetic acid and centrifuged at 12,000 × g for 15 min. Aliquots (20 μl) of the supernatants were removed and applied to phosphocellulose paper (Whatman P-81 paper, VWR). After three washes in 75 mM H₃PO₄ and one wash in acetone, the amount of [γ-³²P]ATP retained was determined by scintillation counting. ERK MAP kinase activation assays were performed as previously detailed (12), or the alkaline phosphatase activity was determined colorimetrically with the Western Blue Stabilized Substrate from Promega (Madison WI).

**Confocal Microscopy**—Differentiated 3T3-L1 cells were replated on Lab-Tek Permanox slides, allowed to attach to the substrate for 6 h, and grown an additional 18 h in fresh medium. Cells were preincubated for 1 h with 40 μM H89, 40 μM PD098059, or vehicle as described for the lipolysis experiments. Following the addition of CL (100 nM, 15 min), cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature, followed by three washes with PBS. The slides were incubated in PBS containing 5% goat serum, 1% BSA, and 0.05% Triton X-100 for 1 h at room temperature, followed by 2 h with chicken anti-rat HSL antisera (18). After three additional washes with PBS, the slides were incubated an additional 2 h with goat anti-chicken Alexa Fluor594 (Molecular Probes). The slides were covered with glass coverslips, mounted using Vectashield mounting medium from Vector Laboratories (Burlingame, CA), and visualized by laser scanning confocal microscope (Zeiss, Thornwood, NY) at 585 nm.

**RESULTS**

When we reported that the β₃AR in adipocytes is coupled to the stimulation of both PKA and ERK (12) we proposed that one consequence of this event could be a convergence of these path-
ways on lipolysis or on brown fat thermogenesis. We could detect no role for ERK in the \(\beta_3\)AR-stimulated increase in \(Ucp1\) gene expression in brown adipocytes (16). Therefore, we studied whether ERK, when activated through the \(\beta_3\)AR, was involved in the acute stimulation of lipolysis in cultured white adipocytes. In the first set of experiments we characterized the relative activation of PKA and ERK in response to selective \(\beta_3\)AR stimulation. Differentiated 3T3-L1 adipocytes were incubated with increasing concentrations of the \(\beta_3\)AR-selective agonist CL or NE (the latter in the presence of 10 \(\mu M\) prazosin, 10 \(\mu M\) yohimbine, and 0.1 \(\mu M\) propranolol, conditions that will only allow stimulation of \(\beta_3\)AR), and kinase activities were measured. Fig. 1A shows that ERK activation, measured as phosphorylation of the kinase by Western blotting (18), was increased 3-fold by both agonists and, as expected, more potently by CL (EC\textsubscript{50}, 14 nM) than by NE (EC\textsubscript{50}, 109 nM). A similar approach was used for the activation of PKA, as measured by \textit{in vitro} phosphorylation of Kemptide (17). As shown in Fig. 1B, CL was also more potent (EC\textsubscript{50}, 0.4 nM) than NE (EC\textsubscript{50}, 6 nM) at stimulating the catalytic activity of PKA. Therefore, these results indicate that lower concentrations of both agonists favor the activation of PKA as compared with ERK. This suggests that, although phenethanolamine agonists like CL interact with additional residues of \(\beta_3\)AR not involved in catecholamine binding (19), both CL and NE seem to equally activate ERK. Fig. 1C shows that the dose-response relationship for the stimulation of lipolysis (EC\textsubscript{50} of 0.1 and 6 nM for CL and NE, respectively) was best correlated with their ability to stimulate PKA. Nevertheless, the ability of \(\beta_3\)AR to stimulate both signaling pathways, and the clear capacity of ERK to stimulate lipolysis in certain situations, required that we fully characterize these two kinase cascades for their involvement in lipolysis selectively stimulated through the \(\beta_3\)AR. Therefore, we first confirmed the selectivity of CL toward the \(\beta_3\)AR at submicromolar concentration. As shown in Fig. 1D, 0.1 \(\mu M\) propranolol, a \(\beta_3\)/\(\beta_2\)AR antagonist, was unable to influence the EC\textsubscript{50} value of CL toward lipolysis (0.3 \textit{versus} 0.1 nM), substantiating that CL acts solely through the \(\beta_3\)AR.

The approach in the next series of experiments was to empirically define the minimal concentrations of the PKA inhibitor H89 and the MEK inhibitor PD098059 necessary for complete inhibition of their respective kinases. As shown in Fig. 2A, PKA activity in response to 100 nM CL was inhibited by H89 in a dose-dependent fashion, with elimination of all kinase activity at a concentration of 40 \(\mu M\). Although increasing concentrations of H89 led to a progressive inhibition of lipolysis (Fig. 2B), note that 21 ± 6% of \(\beta_3\)AR-stimulated lipolysis persisted despite the complete blockade of PKA activity. This result strongly implicated the existence of an alternative signaling pathways.
route to lipolysis by β3AR. Equally significant were the results of reciprocal experiments in which a dose-response curve for CL was performed in the presence of a fixed concentration (40 μM) of H89. Despite the absence of PKA activity (Fig. 2C), a small but significant (p < 0.05) increase in lipolysis (26 ± 5% of the control CL dose-response curve) was measured (Fig. 2D). Interestingly, the potency of CL for this PKA-independent lipolytic activity (EC50, 39 nM) corresponds quite well to the EC50 for CL-stimulated ERK activation estimated in Fig. 1A (14 nM), suggesting its participation in the lipolytic response to β3AR. Because of the possibility that the effects of H89 might be attributed to inhibition of other H89-sensitive kinases (20), we tested the competitive cAMP analog and more specific PKA inhibitor Rp-cAMPS on the lipolytic response of the 3T3-L1 adipocytes (21). As illustrated in Fig. 2E, Rp-cAMPS inhibited 64 ± 6% of CL-promoted lipolysis. This persistent residual activity in the presence of these inhibitors supports the interpretation that there is a PKA-independent component in the lipolytic response to catecholamine.

To evaluate the roles of the ERK pathway in β3AR-regulated lipolysis and characterize the conditions under which it occurs, we employed the same experimental strategy, examining the effect of increasing concentrations of PD098059 (PD) on ERK phosphorylation and glycerol release. As shown in Fig. 3A, PD progressively diminished CL-dependent levels of phospho-ERK to basal values at a concentration of 40 μM. Similarly, CL-stimulated lipolysis was reduced by 27 ± 4% by PD (Fig. 3B). Finally, under conditions where CL-stimulated ERK activation was completely prevented with 40 μM PD (Fig. 3C), again 26 ± 5% of total CL-stimulated lipolysis could be inhibited (Fig. 3D).

To test the hypothesis that ERK plays a physiological role in vivo, we measured plasma glycerol levels following CL injection (3 mg/kg) with or without prior PD injection (10 mg/kg). Because MEK/ERK are broadly expressed in tissues, the effect of PD is obviously not restricted to adipose tissue. However, β3ARs are expressed only in adipose tissue, so any increase in plasma glycerol after CL injection reflects adipose tissue lipolysis. The results shown in Fig. 3E are suggestive of a role for ERK in lipolysis in vivo. Although PD alone increased the basal glycerol level by 45 ± 28% (p < 0.01), it nevertheless was able to blunt the CL-dependent increase by 53 ± 7% (p < 0.001).
tion of \( \beta_3 \)AR, ERK activation might contribute to the translocation of HSL. Using confocal microscopy to visualize HSL, 15 min after addition of 100 nM CL, there was a robust redistribution of HSL from a diffuse cytoplasmic localization (Fig. 5A) to a peri-vacuolar pattern around the lipid inclusion (Fig. 5B). HSL translocation was blocked completely by 40 \( \mu \)M H89 (Fig. 5D), but not by 40 \( \mu \)M PD (Fig. 5F), a concentration that was sufficient to completely prevent ERK activation.

In earlier work on the activation of ERK by the \( \beta_3 \)AR we described an essential role for a direct interaction between proline-rich intracellular portions of the receptor and the cytosolic tyrosine kinase Src (13). At that time, several of the more detailed mechanistic studies were not conducted in adipocytes and no clear functional role was identified. Therefore we directly tested the hypothesis in adipocytes that Src activity and one of its downstream targets, the EGFR, may not only be involved in ERK activation but might also mediate the increase in lipolysis. Fig. 6A shows that inhibition of Src kinase activity with the relatively specific inhibitor PP2 (23) or inhibition of the EGFR kinase with AG1478 (24) totally inhibits ERK activation by CL. Interestingly, these two inhibitors are also able to partially block lipolysis and do so to an extent equivalent to PD (Fig. 6B). Because of the apparent similarity of these effects, we evaluated the effects of Src and EGFR kinase inhibition on the residual H89-insensitive lipolysis. Fig. 6, C and D, clearly illustrates that the PKA-independent portion of CL-induced lipolysis is a Src- and EGFR-dependent process. Although both PP2 and AG1478 are highly specific chemical inhibitors of their target kinases, we used siRNA approaches to confirm that the Src and EGFR pathways are upstream of ERK and lipolysis. As shown in Fig. 6E, knock down of Src expression was virtually complete and for EGFR, which in these cells is modestly expressed, siRNA efficiency was >70%. In this figure we also show that ERK activation by CL was eliminated when the expression of these proteins was suppressed. Functionally, this abrogation of ERK activity was accompanied by an essentially complete disappearance of the portion of lipolysis that was CL induced and H89 insensitive (Fig. 6E).

To test the role of Src and ERK in lipolysis by another approach independent of adrenergic signaling through the \( \beta_3 \)AR, we chose to treat adipocytes with the steroid estradiol (E2). There were two reasons for choosing E2. First, it has been shown to acutely activate ERK in a Src-dependent fashion in adipocytes (25). Second, E2 was described as eliciting an acute lipolytic response (26). As shown in Fig. 7A, ERK activation is dependent not only on Src kinase activity but also on EGFR kinase activity. Perhaps equally important, the results shown in Fig. 7B imply that the activation of lipolysis by E2 is dependent on Src, EGFR, and ERK activity.

**DISCUSSION**

Catecholamines are the major regulators of triacylglycerol hydrolysis in adipocytes, and this lipolytic process is mediated by the \( \beta_3 \)ARs (27). \( \beta_3 \)AR signaling is the principal physiological pathway stimulated when free fatty acids are needed, and its actions are blunted by insulin in the postprandial period. It is now recognized that \( \beta_3 \)ARs can couple to \( G_s \) and \( G_i \), which leads to the consequent activation of the PKA and ERK pathways, respectively (28). In the case of \( \beta_3 \)AR in particular, which is expressed solely in adipocytes, it is constitutively coupled in a dual manner to \( G_s \) and \( G_i \) without the need for regulatory phosphorylation (12, 29, 30). This unique feature of \( \beta_3 \)AR signaling has been of interest to us because of the unusually powerful thermogenic response that can be elicited by agonists that selectively target this receptor (31). Thus, we have
sought to understand some of the physiological consequences of ERK activation by β3AR.

Although the mechanisms responsible for lipolysis by catecholamines are not as clear as they once seemed to be, PKA phosphorylation of perilipin and HSL are undoubtedly decisive events (32, 33). Phosphorylation of HSL by ERK has also been described (14) and shown to be involved in hormone stimulation of lipolysis, at least for endothelin-1 and α-melanocyte-stimulating hormone (34, 35). Mechanistically, in vitro studies using cholesterol-ester as a substrate show that ERK-dependent HSL phosphorylation may increase its catalytic activity (14). However, it does not seem to affect its catalytic activity toward triacylglycerol. Additionally, as we show here, inhibition of ERK does not prevent HSL translocation to the lipid droplet. Therefore, the exact nature of the ERK substrates involved in lipolysis remains under investigation.

In the present study, we have empirically determined the conditions under which PKA and ERK contribute to β3AR-regulated lipolysis. We also determined that this regulation is operating in vivo as well. An additional objective, which arose from our previous demonstration of a β3AR-Src interaction (13), was to test the hypothesis that the β3AR-promoted ERK activation, as well as lipolysis, involves Src and EGFR kinase activities. Our results establish that Src, EGFR, and ERK kinase activities are responsible for 20–25% of β3AR-stimulated lipolysis.

FIGURE 4. Both PKA and ERK are responsible for maximal lipolytic activity by β3AR. Differentiated 3T3-L1 cells were prepared as described in Fig. 1. A–C, cells were pretreated or not with H89 (40 μM), PD098059 (40 μM), or both for 1 h, followed by the addition of CL316,243 (1 or 100 nM). Lipolysis (A), PKA catalytic activity (B), or ERK activation (C) was measured as detailed under “Experimental Procedures.” Results are from four (A) or two (B, C) independent experiments.

FIGURE 5. Translocation of HSL in response to β3AR stimulation requires PKA catalytic activity but not ERK activation. Differentiated 3T3-L1 cells were preincubated with vehicle (A, B), 40 μM H89 (C, D), or 40 μM PD098059 (E, F) for 1 h prior to the addition of vehicle (A, C, E) or 100 nM CL (B, D, F) for 15 min. The slides were processed as described under “Experimental Procedures”, followed by 2 h of incubation with chicken anti-rat HSL antisera and 2 h with goat anti-chicken Alexa Fluor594 (Molecular Probes). Visualization was made on a laser scanning confocal microscope (Zeiss, Thornwood, NY) set at 585 nm. The bar in panel A indicates a 10-μm distance.

3 B. Danielsson and C. Holm, unpublished observation.
cells were prepared as described in Fig. 1. A and B, cells were pretreated or not with H89 (40 μM), PP2 (10 μM), AG1478 (1 μM), or PD098059 (40 μM) for 1 h, followed by the addition of CL316,243 (100 nM). ERK activation (A) or lipolysis (B) was measured as detailed under “Experimental Procedures.” Results are from three independent experiments. C and D, cells were pretreated with H89 (40 μM) in association or not with PP2 (10 μM), AG1478 (1 μM), or PD098059 (40 μM) for 1 h, followed by the addition of CL316,243 (100 nM). ERK activation (C) or lipolysis (D) was measured as detailed under “Experimental Procedures.” Results are from three independent experiments. E, cells were pretreated for 48 h with siPORT Lipid alone (Control), scramble, EGFR, or Src siRNA. ERK activation and lipolysis were determined as detailed under “Experimental Procedures.” *, p < 0.05, **, p < 0.01, and ***, p < 0.001 for the effect of CL316,243. +, p < 0.05, ++, p < 0.01, and ++++, p < 0.001 for the effect of the inhibitors.

FIGURE 6. Contribution of Src, EGFR, and ERK to β3AR agonist-stimulated lipolysis. Differentiated 3T3-L1 cells were prepared as described in Fig. 1. A and B, cells were pretreated or not with H89 (40 μM), PP2 (10 μM), AG1478 (1 μM), or PD098059 (40 μM) for 1 h, followed by the addition of CL316,243 (100 nM). ERK activation (A) or lipolysis (B) was measured as detailed under “Experimental Procedures.” Results are from three independent experiments. C and D, cells were pretreated with H89 (40 μM) in association or not with PP2 (10 μM), AG1478 (1 μM), or PD098059 (40 μM) for 1 h, followed by the addition of CL316,243 (100 nM). ERK activation (C) or lipolysis (D) was measured as detailed under “Experimental Procedures.” Results are from three independent experiments. E, cells were pretreated for 48 h with siPORT Lipid alone (Control), scramble, EGFR, or Src siRNA. ERK activation and lipolysis were determined as detailed under “Experimental Procedures.” *, p < 0.05, **, p < 0.01, and ***, p < 0.001 for the effect of CL316,243. +, p < 0.05, ++, p < 0.01, and ++++, p < 0.001 for the effect of the inhibitors.

Dual Regulation of Lipolysis by PKA and ERK

yis, independent of PKA activity. Additionally, we found that E2-stimulated lipolysis, although quite modest, relies solely on this PKA-independent pathway. By comprehensive examination of the β3AR-dependent activation of PKA, ERK, and lipolysis, we found that ERK activation and its contribution to lipolysis occurs only at maximally effective agonist concentrations. This dose-dependent distinction in the pattern of activation of these two kinase pathways could be a consequence of differential interaction of the β3AR with Gs and Gq, tending to favor Gq coupling (12). Therefore we can hypothesize that under physiological conditions the ERK pathway will be recruited in reaction to high sympathetic nervous system activity such as during cold exposure or fasting (36, 37). Interestingly, results in vivo suggest that pharmacological stimulation of lipolysis through the β3AR also has a significant ERK-dependent component. While the effects of PD in vivo are without a doubt ubiquitous, the high specificity of CL for the β3AR and the restricted expression of this receptor to adipose tissue provide support for such an interpretation.

The ERK-dependent component of the lipolytic response to CL and E2 was detected in less than 60 min, within a reasonable physiologic time frame. This relatively acute effect contrasts with the much slower kinetics (6–48 h) required to achieve a significant degree of lipolysis when adipocytes are exposed to cytokines such as tumor necrosis factor α (38, 39). This faster time frame to stimulate lipolysis is consistent with other observations with endothelin-1, also shown to be mediated by ERK (35). Interestingly, endothelin-1 and E2 share with CL the ability to activate ERK through a G1- and Src-dependent transactivation of EGFR (25, 40–43). Therefore, in adipocytes these pathways that converge on the EGFR seem to be more efficiently targeted to the lipolytic process than is observed for the cytokine receptors. Perhaps these receptors reside together with G1 and EGFR in a privileged membrane microenvironment (caveolae, for example) that favors a fast coupling to some component(s) of the lipolytic apparatus also present in this locus, although this is just speculation. Tumor necrosis factor α, on the other hand, seems to be coupled to lipolysis indirectly through an ERK-dependent change in the expression of perilipin, which could explain the lag before the appearance of the effect (44).

At low β3AR agonist concentrations, the receptor couples to Gs and activates adenylyl cyclase- and PKA-dependent lipolysis. At higher, but still nanomolar, concentrations the receptor interacts with Gs and recruits Src, and both Src and EGFR activities are then required to promote ERK-dependent lipolysis. The fact that Src interacts directly with the β3AR (13) suggests that it is a proximal event, and the inhibitory effect of PP2 on ERK activity shows that it is upstream of the MAP kinase. This pivotal position for Src is not unexpected because Src can phosphorylate the EGFR at two tyrosine residues and Tyr-845 phosphorylation promotes EGFR activity (45). Additionally, Src has been shown to be involved in autocrine transactivation of the EGFR (46).

Altogether, this study characterizes the conditions in which the ERK pathway contributes to the lipolytic process and suggests that it will occur principally under conditions of high sym-
A

|     | Cont | H89 | PP2 | AG | PD |
|-----|-----|-----|-----|----|----|
| E2  | -   | +   | -   | +  | -  |
| P-ERK1/2 | +  | -   | +   | +  | -  |
| ERK1/2 | +  | -   | +   | +  | -  |

B

![Graph showing the contribution of Src, EGFR, and ERK to E2-stimulated lipolysis.](image)

FIGURE 7. Contribution of Src, EGFR, and ERK to E2-stimulated lipolysis. Differentiated 3T3-L1 cells were prepared as described in Fig. 1 except that they were incubated overnight in serum-free medium. A and B, cells were pretreated or not with H89 (40 μM), PP2 (10 μM), AG1478 (1 μM), or PD098059 (40 μM) for 1 h, followed by the addition of E2 (10 nM). ERK activation (A) or lipolysis (B) was measured as detailed under "Experimental Procedures." Results are from three independent experiments. +, p < 0.05 for the effect of the inhibitors.

pathetic nervous system activity and/or in the immediate proximity of the nerve ending. Although we focused on βAR in these studies, there may be wider implications for the role of ERK in lipolysis because βAR and β2AR are also expressed in adipocytes and there is much evidence, particularly for β2AR, that it associates indirectly with Src through the binding of β-arrestin (47), an event that also leads to ERK activation (48). Moreover, in human adipocytes it is debated whether ERK contains low (49) or no (50) β3AR, while expressing significant levels of β1AR and β2AR (22). The possible contribution of βAR-stimulated ERK to lipolysis or other catecholamine-regulated metabolic activities in human adipocytes deserves further exploration.

Acknowledgments—We thank Drs. Tonya Martin and Wenhong Cao for helpful discussions and comments on the manuscript. We also thank Drs. Andrew Greenberg, Sandra Souza, and Gabriel E. Guzman for discussions and preliminary studies on perilipin.

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