Original Article

Adipose Cells Induce Phospho-Thr-172 AMPK Production by Epinephrine or CL316243 in Mouse 3T3-L1 Adipocytes or MAPK Activation and G Protein-Associated PI3K Responses Induced by CL316243 or Aluminum Fluoride in Rat White Adipocytes

(aluminum fluoride / AMP-activated protein kinase / CL316243 / epinephrine / G-protein subunits / mitogen-activated protein kinase / mouse 3T3-L1 adipocytes / phosphatidylinositol 3-kinase / rat white adipocytes)

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Abstract. Responses of adipose cells to adrenoceptor regulation, including that of β-adrenoceptor (AR), and the signalling machinery involved in these responses are not sufficiently understood; information that is helpful for elucidating the adrenoceptor (adrenergic and β-AR)-responsive machinery is insufficient. We examined phospho-Thr-172 AMPK production in mouse-derived 3T3-L1 adipocytes treated with epinephrine or CL316243 (a β_3-AR agonist) for 15 min. We also examined MAPK activation or G protein-associated PI3K activation or -associated PI3K p85 complex formation in rat epididymal (white) adipocytes treated with CL316243 for 15 min or aluminum fluoride (a G-protein signalling activator) for 20 min. Furthermore, we examined the effect of PTX (a trimeric G-protein inactivator) on p85 complex formation induced by aluminum fluoride treatment. Western blot analysis revealed that epinephrine or CL316243 treatment increased the phospho-Thr-172 AMPK (an active form of AMPK) level in 3T3-L1 adipocytes. Activated kinase analysis with a specific substrate showed that CL316243 or aluminum fluoride treatment activated MAPK in rat adipocytes. Immunoprecipitation experiments with a G-protein β subunit (G_β) antibody showed that treatment of rat adipocytes with CL316243 activated PI3K and increased the PI3K p85 level in the G_β antibody immunoprecipitates. Such an increase in the p85 level was similarly elicited by aluminum fluoride treatment in a PTX-sensitive manner. Our results provide possible clues for clarifying the signalling machinery involved in adrenoceptor responses, including those of β_3-AR, in mouse-derived adipocytes and rat white adipocytes. Our findings advance the understanding of responses to adrenoceptor regulation in adipose cells and of the cellular signalling machinery present in the cells.

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Abbreviations: AMP – adenosine monophosphate, AMPK – AMP-activated protein kinase, ATP – adenosine triphosphate, BRL37344 – sodium 4-[(2R)-2-[[(2R)-2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxyacetate, cAMP – adenosine 3',5'-cyclic monophosphate, CL316243 – disodium (*R*,*R*)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate, ERK – extracellular signal-regulated kinase, Gi – inhibitory G protein, GLUT – glucose transporter, Gs – stimulatory G protein, GTP – guanosine triphosphate, GTPγS – guanosine 5'-[γ-thio]triphosphate, HSL – hormone-sensitive lipase, ISO – isoproterenol, LKB – liver kinase B, MAPK – mitogen-activated protein kinase, PI3K – phosphatidylinositol 3-kinase, PTX – pertussis toxin.

Introduction

Exposure of adipose cells to adrenergic stimulation induces cellular responses, including molecular phosphorylation, lipolysis and glucose transport. Adrenoceptor signalling is implicated in the development of adipocyte disorders and can be targeted to improve adi-

pocyte dysregulation (Arner and Hoffstedt, 1999). However, adrenergic responses in adipocytes and the signalling machinery present in the cells, including signalling machinery that induces adrenoceptor responses, have not been fully elucidated.

Treatment of rat adipocytes, which mainly express β_1- and β_3-adrenoceptors (ARs), with adrenaline, a stress-responsive hormone, for 10 min activates AMP-activated protein kinase (AMPK) in a β-AR-dependent manner (Koh et al., 2007). AMPK targeting experiments showed that on treatment of rat or mouse adipocytes with a non-selective β-AR activator (for 20 min to 1 h), the lipolytic and anti-lipolytic effects involved activation of AMPK (Yin et al., 2003; Daval et al., 2005). However, the function of AMPK in response to adrenaline in adipocytes and adrenaline-responsive signalling to AMPK in the cells are not well understood, although an AMPK inhibitor partially blunts lipolysis induced by adrenaline in rat adipocytes (Koh et al., 2007). Treatment of rat adipocytes with a β_3-AR agonist, BRL37344, for 1 h activates AMPK (Daval et al., 2005), but this agonist was reportedly more effective than another β_3-AR agonist, CL316243 (CL), in tissues expressing β_1- or β_2-AR (Bloom et al., 1992). Mouse-derived 3T3-L1 (L1) adipocytes express mainly β_2- and β_3-ARs (Monjo et al., 2005) and preferentially bind to epinephrine (EPI, adrenaline) (Lai et al., 1982), and 15-min treatment with CL induced a response similar to that induced by EPI in L1 adipocytes (Ohsaka and Nishino, 2013). Long-term treatment of C57BL/6 mice with CL activates AMPK in adipose tissues (Mulligan et al., 2007). However, it is not clear whether short-term treatment with CL or adrenaline activates AMPK in mouse adipocytes, although the effect of 1-h EPI treatment on AMPK activation (phospho-Thr-172 AMPK production) in L1 adipocytes has been examined (Watt et al., 2006).

Treatment of mouse L1 adipocytes with CL for 15 min activates ERK (MAPK) (Robidoux et al., 2006). MAPK inactivation partially suppressed CL-induced lipolysis in L1 adipocytes (Greenberg et al., 2001; Robidoux et al., 2006), and GTP-binding (G) protein regulated MAPK activation in mouse adipocytes (Soeder et al., 1999; Cao et al., 2000). Mouse adipocytes express two types of β_3-AR (β_{3a}- and β_{3b}-ARs). The C-terminal cytoplasmic tail of the mouse β_{3a}-AR is similar to that of the rat β_3-AR (Sato et al., 2005). Examination of MAPK activation following β_3-AR or G-protein signalling in rat adipocytes provides information that is helpful for clarifying which type of β_3-AR mediates mouse adipocyte responses to β_3-AR activation and what signalling machinery activates MAPK in rat adipocytes. Such examination is also helpful for clarifying the signalling machinery involved in rat β_3-AR-mediated lipolysis and rat MAPK-dependent receptor responses. The effects of the selective β_3-AR agonist CL or G-protein signalling on MAPK activation in rat adipocytes remain unclear.

MAPK phosphorylates a low-molecular-weight protein *in vitro* (Haystead et al., 1994). In rat (epididymal) adipocytes, this low-molecular-weight protein is phosphorylated via β-AR following 10-min adrenergic stimulation (Diggle and Denton, 1992); such phosphorylation induced by β-AR activation is induced in cells treated with insulin, a hormone that activates MAPK (Sevetson et al., 1993). The signalling machinery involved in phosphorylation of the low-molecular-weight protein following β-AR (non-selective β-AR) activation in rat adipocytes is poorly understood. It is not known whether MAPK activation is regulated by β-AR signalling in rat white adipocytes.

Treatment of rat (white) adipocytes with CL for 10 min induces protein kinase activation in a wortmannin (WT)-sensitive manner (Zmuda-Trzebiatowska et al., 2007). WT inactivates several molecules (Knight et al., 2006), including phosphatidylinositol kinase. However, previous research has not investigated the rat adipocyte molecules involved in the WT-sensitive response to CL. The CL-responsive kinase is activated by 3-phosphoinositide-related molecules (Walker et al., 1998), and WT was shown to directly bind to some PI3Ks that are activated by the G-protein β γ ($G_{\beta\gamma}$) subunits (Kurosu et al., 1995). Additionally, when rat adipocytes express 3-phosphoinositide-dependent protein kinase-1, MAPK is activated (Sajan et al., 1999). The effect of CL on the PI3K pathway and the signalling that induces this effect in rat adipocytes are poorly understood.

PI3K p85/p110 activity is sensitive to WT and is also increased by $G_{\beta\gamma}$ *in vitro* (Hazeki et al., 1998). The PI3K p85/p110 dimer can be immunoprecipitated with an antibody to insulin receptor substrate-1 (IRS-1) and activated following treatment of rat adipocytes with insulin (Kelly and Ruderman, 1993). Expression of a constitutively active form of PI3K p110 in rat adipocytes induces translocation of a glucose transporter (GLUT) to the plasma membrane (PM) (Tanti et al., 1996). The number of PM GLUT molecules is increased by a non-selective β-AR activator for 30 min in rat adipocytes (Shirakura et al., 1990). PM GLUT translocation is also induced by treatment of the cells for 20 min with a GTP analogue that activates both trimeric and small G proteins (Baldini et al., 1991). This treatment (for 15 min) activated membrane PI3K in rat adipocytes (Standaert et al., 1998). The machinery that mediates the β-AR activation-caused PM GLUT induction and the GTP analogue-regulated PI3K activation and subsequent response in the cells is not well understood. A 10-min treatment of rat adipocytes with a fluoride ion (the stimulatory effects of which are blocked by a chelator of the aluminum ion) (Suzuki et al., 1992), which is a trimeric G-protein signalling activator, induces a response (glucose transport) similar to that induced by β-AR activators, including CL (Ohsaka et al., 1998). Treatment with a trimeric G-protein inactivator, PTX, alters GTP-regulatory signalling in rat adipocytes (Chaudhry et al., 1994). It is unclear whether PI3K p85/p110 and $G_{\beta\gamma}$ are involved in β-AR or trimeric G-protein signalling in rat adipocytes.

To investigate adrenoceptor (adrenergic and β-AR) responses in adipocytes and to obtain information help-

ful for elucidating the adrenoceptor-responsive machinery in the cells, we treated mouse L1 adipocytes with EPI or CL for 15 min or rat epididymal white adipocytes with CL or G-protein signalling activator aluminum fluoride for 15 or 20 min and examined the phospho-Thr-172 AMPK level in L1 adipocytes or MAPK activation in rat adipocytes. In addition, rat adipocytes were treated with CL for 15 min, and activation of PI3K and association of PI3K p85/p110 with G-protein subunits were examined by immunoprecipitation with an antibody to the G-protein β (G_β) subunit and by detection of PI3K p85. We also examined, by immunoprecipitation, the effect of trimeric G-protein inactivation by PTX on the 20-min aluminum fluoride treatment-induced association of PI3K p85 with the G_β subunit.

Material and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Bovine serum albumin (BSA, fatty-acid free), CL, EPI, insulin, 3-isobutyl-1-methyxanthine (IBMX), leupeptin, Nonidet P (NP)-40, phenylmethylsulphonyl fluoride (PMSF), sodium dodecyl sulphate (SDS), and Tris were purchased from Sigma-Aldrich Co. (St. Louis, MO). Aprotinin was obtained from Roche Diagnostics (Indianapolis, IN). Collagenase type I and PTX were obtained from Worthington Biochemical Co. (Freehold, NJ) and Kaken Pharmaceutical Co. (Tokyo, Japan), respectively. Anti-phospho-AMPK (Thr-172) rabbit antibody and anti-PI3K (p85) and anti-IRS-1 rabbit antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA) and Upstate Biotechnology Inc. (Lake Placid, NY), respectively. Anti-G_β rabbit antibody (KT, MS, RA or SW, for a different G_β peptide; Murakami et al., 1992) was a kind gift from Dr. T Murakami (Department of Clinical Biochemistry, Hokkaido University School of Medicine, Japan). Protein G-Sepharose 4 Fast Flow was purchased from GE Healthcare Life Sciences (Piscataway, NJ). Phosphatidylinositol (PI), Silica gel 60 plate, and [γ-^{32}P]ATP were purchased from Serdary Research Laboratories (Ontario, Canada), Merck Millipore (Darmstadt, Germany), and Dupont NEN (Boston, MA), respectively. Ethylenediaminetetraacetic acid (EDTA), 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), oil red O, polyacrylamide, sodium chloride, and sucrose were purchased from Nacalai Tesque (Kyoto, Japan). Ethylene glycol tetraacetic acid (EGTA) was from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Aluminum chloride, dithiothreitol (DTT), sodium fluoride, and other chemicals (unless otherwise stated) were purchased from Wako Chemicals (Osaka, Japan).

Treatment of 3T3-L1 adipocytes

3T3-L1 fibroblasts were plated at a density of 3.5×10^4 cells/cm^2 in DMEM supplemented with 10% heat-inactivated foetal calf serum (HyClone, South Logan, UT) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C and were differentiated into adipocytes as described previously (Ohsaka and Nishino, 2013). Adipocyte differentiation was confirmed by staining triacylglycerol droplets in the cells with oil red O and also by examining the signalling pathway downstream of β_3-AR whose expression is induced after differentiation (Monjo et al., 2005); cAMP accumulation was determined by radioimmunoassay using a cAMP assay kit (Yamasa Shoyu Co., Chiba, Japan) following CL treatment of L1 adipocytes. Seven to nine days following the onset of differentiation, L1 adipocytes were treated with or without EPI or CL in Krebs-Ringer HEPES (KRH) buffer containing 1% BSA at 37 °C. Before exposure to agonists, differentiation of cells in each dish was examined by spectrophotometric measurement of lipid dye absorbance at 510 nm (Ramírez-Zacarías et al., 1992). The agonist-treated and -untreated L1 adipocytes were homogenized with a buffer containing 10 μg/ml aprotinin, 1 mM EDTA, 20 mM HEPES (pH 7.4), 1 mM PMSF, and 0.25 M sucrose and then analysed by western blotting.

Treatment of rat adipocytes

Epididymal adipose tissues were isolated from a Wistar-derived strain weighing between 200 and 300 g. Adipocytes were isolated by digesting adipose tissues in a Krebs-Ringer bicarbonate (KRB) buffer containing 3% BSA and 250 U/ml collagenase, as described previously (Ohsaka et al. 1998). Cells suspended in the KRB buffer containing 3% BSA at a final concentration of 10^6 cells/ml were incubated with or without CL, aluminum fluoride, or insulin at 37 °C or pre-treated with PTX before the aluminum fluoride treatment.

Measurement of MAPK activation

MAPK activation was determined by measuring ^{32}P incorporation in a MAPK-specific substrate by using a MAPK enzyme assay system (GE Healthcare Life Sciences), according to the manufacturer's instructions. Rat adipocytes treated or untreated with agents were lysed using a buffer containing 10 μg/ml aprotinin, 2 mM DTT, 2 mM EGTA, 10 μg/ml leupeptin, 150 mM NaCl, 1% NP-40, 1 mM PMSF, and 10 mM Tris (pH 7.4), and the lysates were incubated with [γ-^{32}P]ATP and the assay substrate at 32 °C for 30 min. The reaction was stopped by transferring the reaction mixture onto p81 filter paper (Whatman plc, GE Healthcare Life Sciences). ^{32}P radioactivity incorporated in the substrate was quantified by liquid scintillation counting.

Immunoprecipitation and measurement of PI3K activation

Rat adipocytes were lysed as described previously (Ohsaka et al., 1997). Aliquots of cell lysates were mixed with the indicated antibody that had been pre-adsorbed on protein G-Sepharose and then stirred over-

night at 4 °C. Following centrifugation, immune complexes recovered in the pellet were incubated in a buffer containing PI and [γ-^{32}P]ATP as described previously (Giorgetti et al., 1992) or were analysed by western blotting. Reaction mixtures containing radiolabeled PI were separated by thin-layer chromatography on a Silica Gel 60 plate in a chloroform/methanol/25% NH_4OH/water (100:70:25:15, v/v) mixture. ^{32}P-labled PI was detected and quantified by using a Fuji BAS2000 Bioimaging Analyzer (Fuji Photo Film Co., Tokyo, Japan).

Western blot analysis

Aliquots of L1 adipocyte homogenates or rat adipocyte-derived immune complexes were separated by 10% or 8% SDS polyacrylamide gel electrophoresis and transferred onto a Hybond-enhanced chemiluminescence (ECL) filter (GE Healthcare Life Sciences). The separated proteins transferred onto the filter were incubated for 1−1.5 h with the indicated antibody (1 : 1000). After incubating with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (1 : 2500; Cell Signaling Technology Inc.), the immune complexes were detected on X-ray film (Kodak X-Omat AR; Eastman-Kodak Co., Rochester, NY) by using an ECL detection system (GE Healthcare Life Sciences). The blots on the film were quantified using the Image J program (http://rsb.info.nih.gov/ij/index.html).

Statistical analysis

The results were evaluated by using analysis of variance (ANOVA) with Scheffe's test (for comparisons among multiple groups) and using unpaired Student's *t*-test (for differences between two groups). Statistical significance was defined as $P < 0.05$ and is indicated with an asterisk mark (*). Values are presented as means ± SD of three or four experiments.

Results

Increases in the level of phospho-Thr-172 AMPK in L1 adipocytes treated with EPI or CL

We examined the phospho-Thr-172 AMPK level in L1 adipocytes treated with EPI or CL. The level of phospho-Thr-172 AMPK increased following treatment with EPI or CL (0.01−1 μM) for 15 min (Fig. 1a, b); there was no significant increase in the phospho-AMPK level after treatment with 1 or 0.1 nM of EPI or CL. Prior to treatment with agonists, cells plated on each dish did not exhibit quantitative differences in the levels of cellular lipids (Fig. 1d), which serve as a marker for adipocyte differentiation. Additionally, no variation in the phospho-Thr-172 AMPK level was observed in L1 adipocytes after incubation for 15 min in the absence of agonists (Fig. 1c). Results showing that the levels remained unchanged without agonists were also obtained in other experimental replicates (data not shown). Increased production of cAMP, a key signalling molecule in differentiated L1 adipocytes, as well as induction of lipid droplets (data not shown), was observed in the adipocytes (Fig. 1e).

Induced MAPK activation in rat adipocytes treated with CL

We treated rat adipocytes with CL and examined MAPK activation. Treatment of cells for 15 min with CL (0.01 and 0.1 μM) activated MAPK (Fig. 2a). However, the increase in MAPK activation was not significant in the treatment with 1 nM CL and was lost by increasing the CL concentration to 1 μM (Fig. 2a). MAPK was activated by treatment with insulin (0.7 μM) for 15 min (Fig. 2b). MAPK activation did not vary in cell suspensions incubated without agonists (Fig. 2c).

Induced PI3K activation and PI3K p85 immunoprecipitation with G_β RA antibody in rat adipocytes treated with CL

PI3K was immunoprecipitated with some G_β antibodies in rat adipocytes following treatment with CL for 15 min. Following immunoprecipitation with the G_β RA antibody, treatment of cells with 0.1 μM CL activated PI3K (Fig. 3a, b). However, PI3K activation was not induced following immunoprecipitation with the G_β MS, KT, or SW antibody (Fig. 3a). In accordance with results of previous studies (Giorgetti et al., 1992; Kelly and Ruderman, 1993), rat adipocytes induced PI3K activation following immunoprecipitation with an anti-IRS-1 antibody (Fig. 3b) and the level of PI3K p85 increased in IRS-1 antibody immunoprecipitates (Fig. 3c) in response to insulin. PI3K activation did not vary in experimental replicates incubated in the absence of agonists (Fig. 3b, inset). We examined the effect of CL on the level of PI3K p85 in the G_β RA antibody immunoprecipitates. Treatment of rat adipocytes with 0.1 μM CL for 15 min increased the PI3K p85 level in G_β RA antibody immunoprecipitates (Fig. 3d); treatment with 1 or 10 nM CL did not significantly increase the PI3K p85 level. The PI3K p85 level in G_β RA antibody immunoprecipitates did not change in cells incubated in the absence of CL (data not shown).

Induced MAPK activation and PTX-sensitive immunoprecipitation of PI3K p85 with G_β RA antibody in aluminum fluoride-treated rat adipocytes

We treated rat adipocytes with aluminum fluoride and examined MAPK activation and PI3K p85 level in G_β RA antibody immunoprecipitates. We also pre-treated rat adipocytes with PTX before aluminum fluoride treatment and examined the level of PI3K p85 in the immunoprecipitates. Following treatment of cells with 10 mM aluminum fluoride for 20 min, MAPK was activated (Fig. 4a) and the level of PI3K p85 in G_β RA antibody immunoprecipitates increased (Fig. 4b); these responses were not observed significantly after treatment with

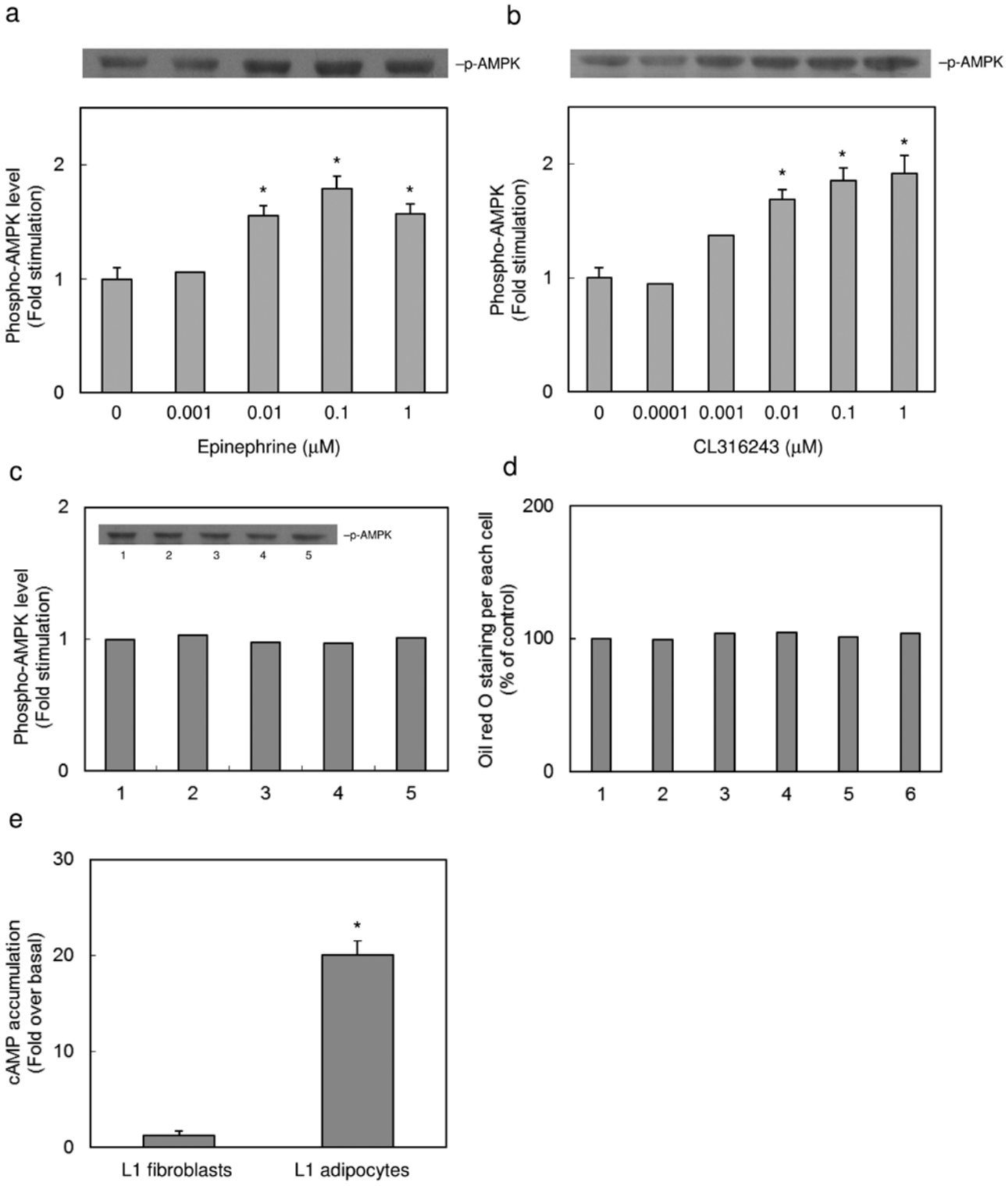

Fig. 1. Effects of epinephrine or CL316243 on phospho-Thr-172 AMPK level in L1 adipocytes
a) L1 adipocytes were treated with the indicated concentrations of **a)** epinephrine (EPI) or **b)** CL316243 (CL) for 15 min. Cell lysates were prepared from treated or untreated cells, and phospho-Thr-172 AMPK levels were determined by western blotting using a phospho-AMPK (Thr-172) antibody. **c)** and insets 1−5 show the phospho-Thr-172 AMPK levels in the cells treated for 15 min without agonists. **d)** shows the amount of oil red O dye staining in each dish of cells prior to treatment with agonists; data are expressed as percentages of values obtained from cells treated for 15 min without agonists. **e)** shows cAMP accumulation in L1 adipocytes or its precursor cells (L1 fibroblasts) treated with 0.1 μM CL for 10 min in the presence of IBMX (a phoshodiesterase inhibitor, 0.3 mM). Blots and insets 1−5 represent typical results (3 or 4 experiments).
*$P < 0.05$ vs. L1 adipocytes not treated with EPI (**a**) or CL (**b**), or vs. L1 fibroblasts (**e**)

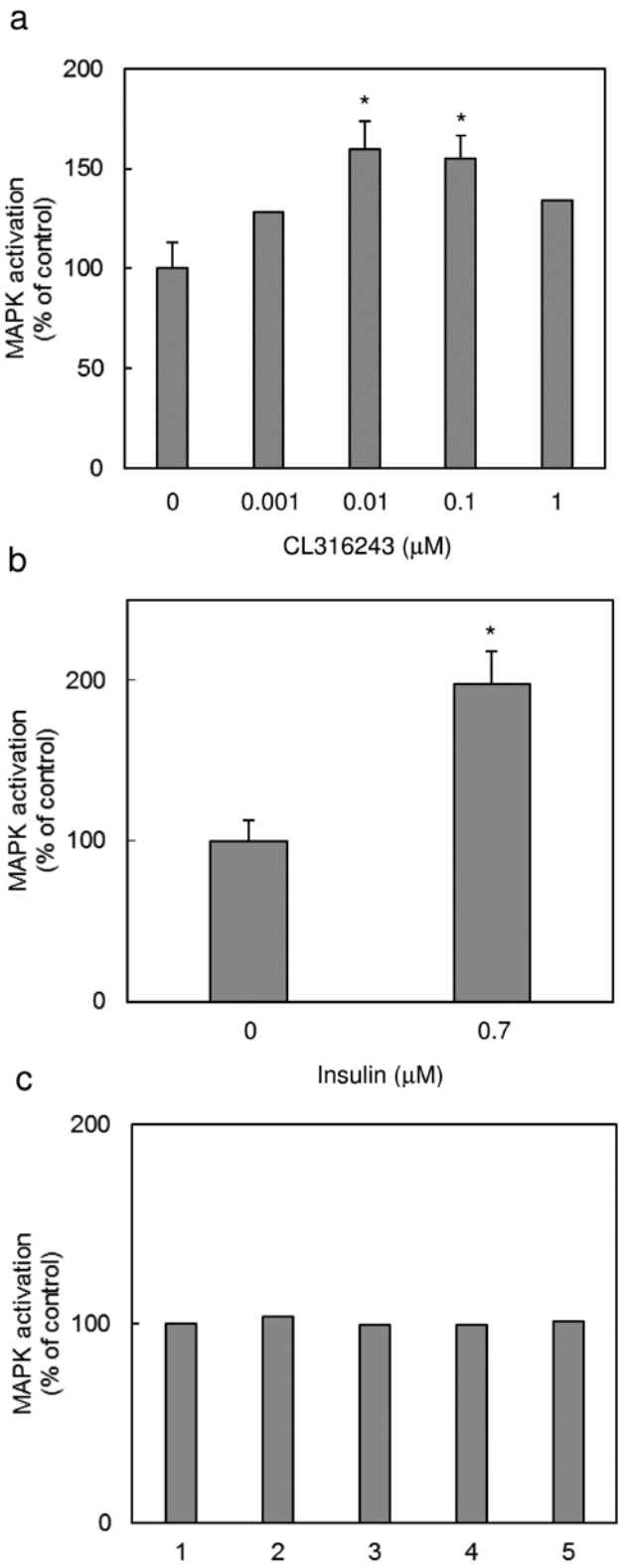

Fig. 2. Effects of CL316243 on MAPK activation in rat adipocytes. Rat adipocytes were treated with the indicated concentrations of CL316243 (CL, **a**) or insulin (**b**) for 15 min. Cell lysates were prepared from cells treated with or without agonists, and MAPK activation was measured. C shows MAPK activation in cells treated for 15 min without agents.
*P < 0.05 vs. cells not treated with CL (**a**) or insulin (**b**)

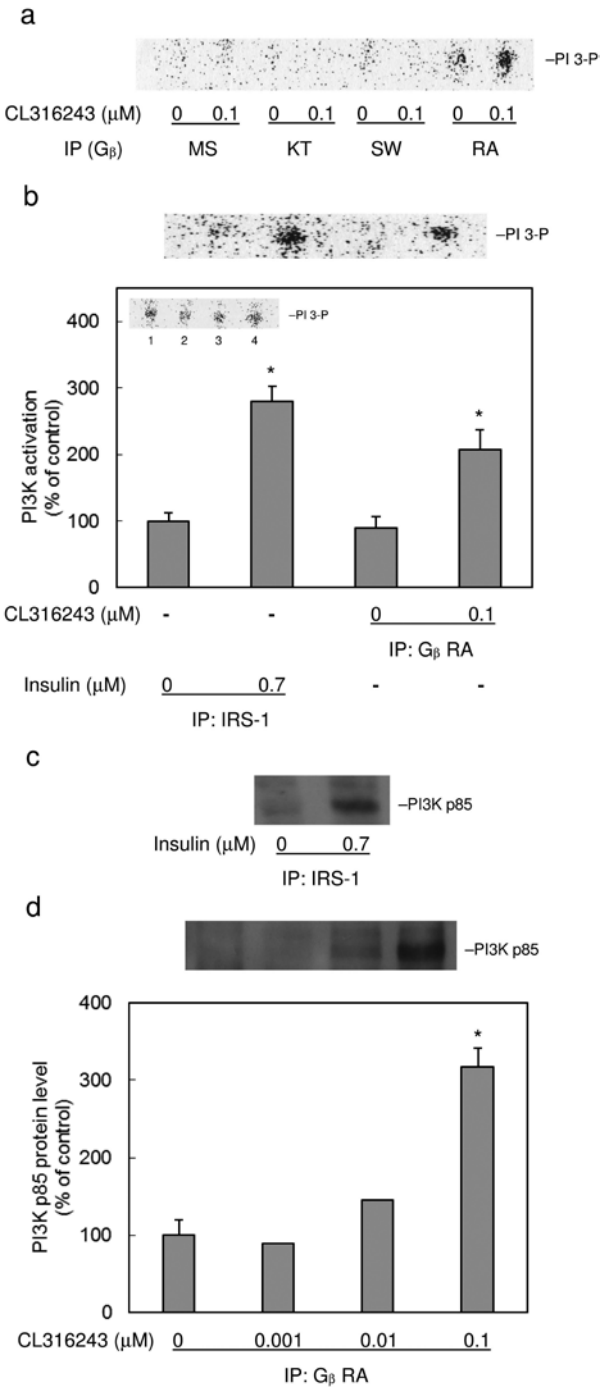

Fig. 3. Effects of CL316243 on PI3K activation following immunoprecipitation with a G_β antibody and on immunoprecipitation of PI3K p85 with the G_β antibody. Rat adipocytes were treated with the indicated concentrations of CL316243 (CL; **a**, **b**, and **d**) or insulin (**b** and **c**) for 15 min. Cell lysates were prepared and then immunoprecipitated (IP) with the indicated G_β antibody (MS (**a**), KT (**a**), SW (**a**), or RA (**a**, **b** and **d**)) or an anti-IRS-1 antibody (**b** and **c**). Immunoprecipitated proteins were used to examine PI3K activation (**a** and **b**) and detection of PI3K by western blotting using a PI3K p85 antibody (**c** and **d**). Insets 1–4 show PI3K activation in cells treated for 15 min without agonists. Autoradiographic images and blots represent typical results (3 or 4 experiments).
*P < 0.05 vs. cells not treated with agonists (**b** and **d**)

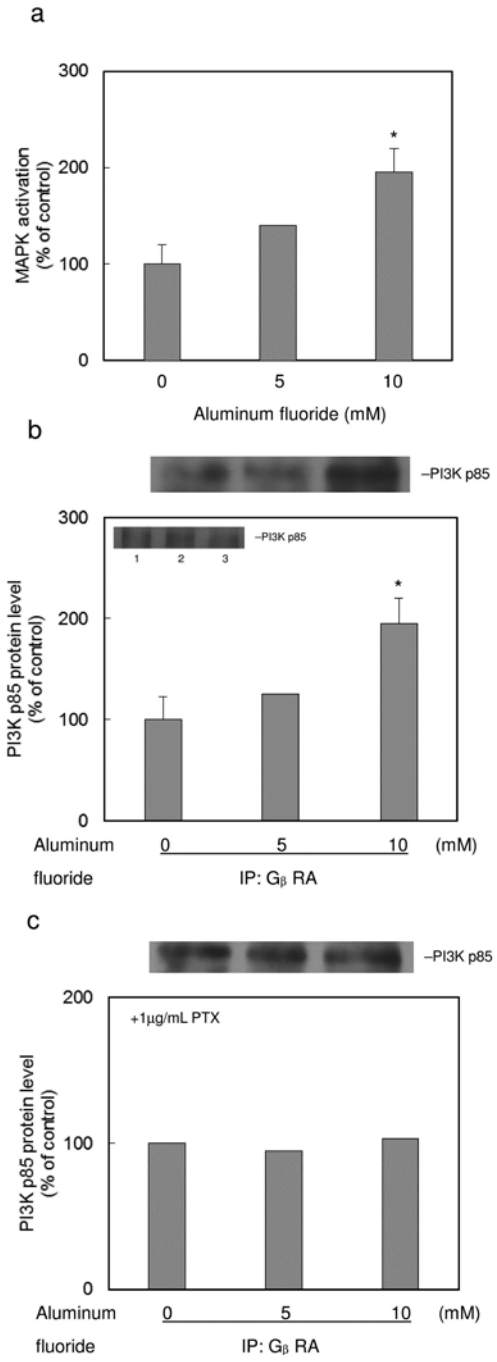

Fig. 4. Effects of aluminum fluoride on MAPK activation and on PI3K p85 immunoprecipitation with a G_β antibody following pre-treatment with or without PTX. Rat adipocytes were treated with the indicated concentrations of aluminum fluoride for 20 min (**a** and **b**) or were pre-treated with PTX for 60 min at 37 °C before the 20-min aluminum fluoride treatment (**c**). Cell lysates were prepared, and MAPK activation was measured (**a**). Prepared cell lysates were also immunoprecipitated (IP) with a G_β RA antibody, and PI3K was detected by western blotting using a PI3K p85 antibody (**b** and **c**). Insets 1−3 show the level of PI3K p85 in G_β RA antibody immunoprecipitates after 20-min treatment without aluminum fluoride. Blots represent typical results (3 or 4 experiments).
*$P < 0.05$ vs. cells not treated with aluminum fluoride (**a** and **b**)

5 mM aluminum fluoride. There was no change in either the MAPK activation level (data not shown) or the PI3K p85 level in G_β RA immunoprecipitates (Fig. 4b, inset) in cell suspensions incubated in the absence of aluminum fluoride. After pre-treatment with PTX for 60 min, the PI3K p85 level in G_β RA immunoprecipitates did not change in cells treated for 20 min with aluminum fluoride (Fig. 4c) and without aluminum fluoride (data not shown).

Discussion

In this study, we observed responses to EPI, CL316243, or aluminum fluoride in L1 adipocytes and rat adipocytes. Adipocyte responses induced by the β_3-AR agonist CL and the G-protein signalling activator aluminum fluoride were similar to those induced by adrenergic stimulation in L1 adipocytes and by β_3-AR stimulation in rat adipocytes. These adipocytes possess cellular machinery, including that mediating adrenergic or β-AR stimulation-induced lipolytic (Chernick et al., 1986) or anti-lipolytic effects, low-molecular-weight protein phosphorylation, PM GLUT induction and glucose transport (Ludvigsen et al., 1980; Shirakura et al. 1990), and G-protein activator treatment-induced PI3K activation and glucose transport-related responses. Aluminum fluoride treatment mimics the signalling events that follow receptor activation; its effects include induction of trimeric G-protein activation and adenylyl cyclase modulation (Sternweise and Gilman, 1982). Phospho-Thr-172 AMPK production is induced in L1 adipocytes by treatment with aluminum fluoride or a non-selective β-AR activator, isoproterenol (ISO), for 15 min (Yin et al., 2003; Ohsaka et al., 2010). Molecular responses to adrenergic stimulation in adipocytes and the signalling machinery included in these responses (machinery present in the cells), including those of β-ARs, are not fully understood. AMPK is activated in rat adipocytes treated with adrenaline or BRL37344. ERK phosphorylation (phospho-tyrosine/threonine ERK(s) production) is induced in mouse adipocytes by 5 or 15-min treatment with CL (Soeder et al., 1999; Cao et al., 2000; Robidoux et al., 2006) and in mouse brown adipocytes by 5-min treatment with BRL37344 (Lindquist et al., 2000). In rat adipocytes, CL treatment activated protein kinase B (PKB) in a WT-sensitive manner (Zmuda-Trzebiatowska et al., 2007) and PTX treatment altered trimeric Gi or Gs signalling (Chaudhry et al., 1994). We found that EPI or CL treatment for 15 min induces phospho-Thr-172 AMPK production in L1 adipocytes and that CL or aluminum fluoride treatment for 15 or 20 min induces MAPK activation in rat white adipocytes. We also found that treatment of the rat white adipocytes with CL for 15 min activates PI3K in immunoprecipitates with a G_β antibody and increases the PI3K p85 level in G_β antibody immunoprecipitates and that this increased p85 level is similarly elicited by aluminum fluoride treatment in rat white adipocytes in a PTX-sensitive manner.

cAMP-dependent kinase was activated and cAMP was produced in L1 adipocytes after CL treatment for 5 (Robidoux et al., 2006) or 10 min (Fig. 1e). CL is thought to activate the adenylyl cyclase system. AMPK is phosphorylated at the Thr-172 residue and activated following treatment with an adenylyl cyclase activator, forskolin, or a cAMP analogue for 5−30 min in L1 adipocytes (Yin et al., 2003). These agents can alter the AMP/ATP ratio and modify an AMPK kinase, LKB1, in the cells (Gauthier et al., 2008). Experiments using LKB1 hypomorphic mice showed that AMPK kinase in adipocytes regulates AMPK phosphorylation at the Thr-172 residue and activation of the AMPK isoforms AMPKα1 and α2 (Gormand et al., 2011). L1 adipocytes expressed these isoforms (Salt et al., 2000). The cAMP content in the cells increases in response to EPI treatment for ≤ 10 min (Elks et al., 1984). EPI or CL treatment may induce production of a phospho-Thr-172 AMPK isoform(s) by altering the AMPK kinase activity and/or the AMP/ATP ratio and activate AMPK.

AMPKα2 knockout (KO) mice develop adiposity (Villena et al., 2004). The size of adipocytes is reduced in AMPKα1 KO mice (Daval et al., 2005). The use of an antagonist has shown that the response to EPI is mediated by signalling through β-ARs in L1 adipocytes (Mulder et al., 2005). Previous studies using ISO in mouse adipocytes have shown both a lipolytic function and an anti-lipolytic function of AMPK; ISO-induced lipolysis is decreased by the expression of a dominant-negative form of AMPKα2 in L1 adipocytes (Yin et al., 2003) and is increased by the expression of a dominant-negative form of AMPKα1 in isolated mouse adipocytes (Daval et al., 2005). EPI-induced phospho-Thr-172 AMPK production would be helpful for working toward elucidation of the function of AMPK in adrenergic stimulation in mouse-derived adipocytes.

β_3-AR mediates an L1 adipocyte response to EPI (10-min treatment) (Mulder et al., 2005). Fifteen-min treatment with ISO induces phospho-Thr-172 AMPK production in L1 adipocytes. EPI may induce phospho-Thr-172 AMPK production via β-ARs, including β_3-AR. Treatment with EPI or ISO for 15−20 min induces lipolysis in L1 adipocytes (Chernick et al., 1986; Yin et al., 2003), and pre-treatment of the cells with an AMPK activator inhibits lipolysis and translocation of the enzyme hormone-sensitive lipase (HSL) to lipid droplets, which are induced by ISO (Daval et al., 2005). The signalling machinery that mediates these adrenoceptor responses is not sufficiently understood. CL-induced phospho-Thr-172 AMPK production may be helpful for examining the signalling machinery involved in EPI- or ISO-induced phospho-AMPK production and also in lipolytic or anti-lipolytic responses to adrenoceptor (adrenergic or β-AR) regulation in L1 adipocytes.

Phosphorylation of ERK(s) occurs in β_3-AR agonist-treated mouse adipocytes and is required for activation of these kinases (Roskoski, 2012). 3-Phosphoinositides are produced upon PI3K activation in a PI3K p85-containing immunocomplex (Kelly and Ruderman, 1993). Expression of 3-phosphoinositide-dependent protein kinase-1 or expression of another 3-phosphoinositide-responsive kinase (Standaert et al., 1997) activates ERK2 in rat adipocytes (Sajan et al., 1999). ERK1 activation induced by $G_{\beta\gamma}$ is impaired in a cell line expressing a dominant-negative mutant of PI3K p85 (Hawes et al., 1996). Rat adipocytes expressed the isoforms ERK1 and 2 (Sevetson et al., 1993); we detected MAPK activation in the cells by insulin treatment, which produces phosphoproteins and activates these ERK isoforms. CL or aluminum fluoride treatment may activate a MAPK isoform(s) through p85 signalling and phosphorylation of MAPK(s) in rat adipocytes.

A receptor-kinase inhibitor of the epidermal growth factor (EGF) receptor impairs the ERK phosphorylation (activation) induced by treatment with 0.1 μM CL for 15 min (non-kinase type receptor stimulation) in L1 adipocytes (Robidoux et al., 2006). The ERK activation in rat adipocytes treated with EGF or insulin (which activates its own receptor kinase) is inhibited by forskolin treatment (Sevetson et al., 1993). PI3K activation in insulin-treated rat adipocytes is suppressed by exposure to a high concentration of cAMP (Ohsaka et al., 1997). Induction of MAPK activation by treatment with low concentrations (0.01 and 0.1 μM) of CL was impaired when the CL concentration was increased to 1 μM. Such inhibition of activation by a high concentration of CL was also observed in another study (Ohsaka et al., 1998) and examination of PI3K activation (data not shown) in ≥ 1 μM CL-treated rat adipocytes. A decreased MAPK response to increasing concentrations of CL in rat adipocytes may be due to negative regulation of the low CL concentration-sensitive signalling to MAPK.

MAPK was activated by CL treatment in rat adipocytes expressing β_3-AR with a C-terminal tail that is similar to that of the mouse β_{3a}-AR. A peptide derived from the C-terminal tail of mouse β_{3a}-AR (but not β_{3b}-AR) alters its own receptor response in a cell line (Sato et al., 2005), which is modulated by PTX pre-treatment. Such a PTX-sensitive response is similarly observed in rat adipocytes (Chaudhry et al., 1994) and mouse 3T3-F442 adipocytes (Soeder et al., 1999); PTX treatment alters CL-induced MAPK activation in 3T3-F442 adipocytes and mouse C3H10T1/2 adipocytes (Cao et al., 2000). The receptors that mediate the CL-induced MAPK activation in mouse-derived adipocytes may include mouse β_{3a}-AR.

Rat adipocytes express G proteins including PTX-sensitive G proteins, such as Gi and its subtypes (Gi1 and 2) (Mitchell et al., 1989) and Gs (Hinsch et al., 1988). β_3-AR can be coupled to Gi and Gs in rat adipocytes (Chaudhry et al., 1994). Aluminum fluoride treatment appears to activate MAPK by these G proteins in rat adipocytes. Aluminum fluoride-induced effects on MAPK activation may be useful for clarifying the signalling machinery involved in CL-induced MAPK activation in rat adipocytes and the signalling machinery that is expressed in the cells.

Lipolysis is induced by CL treatment and also adrenergic stimulation in rat adipocytes (Ohsaka et al., 1998) as well as in L1 adipocytes. Lipolysis induced by adrenaline treatment was not completely suppressed by an AMPK inhibitor in rat adipocytes. Activated MAPK phosphorylated HSL *in vitro* (Greenberg et al., 2001). A 22-kDa protein (PHAS-I, a low-molecular-weight protein) that is phosphorylated by MAPK (Haystead et al., 1994) is phosphorylated following treatment with ISO or adrenergic stimulation (Diggle and Denton, 1992). The machinery that mediates the adrenergic stimulation-induced lipolysis and PHAS-I phoshorylation in rat adipocytes is not well understood. A selective β_3-AR antagonist impairs adrenergic responses in rat adipocytes (Nisoli et al., 1996). CL and aluminum fluoride-induced MAPK activation may be helpful for examining the signalling machinery involved in the lipolytic and phosphorylative responses to adrenoceptor regulation in rat adipocytes.

PTX-catalysed ADP-ribosylation of the α subunit of trimeric G protein, which inactivates this protein, does not occur when the G protein is activated (when the G_α and $G_{\beta\gamma}$ subunits are dissociated) following activation of the G protein-coupled receptor (Katada et al., 1984). In the presence of $G_{\beta\gamma}$, ADP-ribosylation of the G_α subunit induced by PTX is inhibited *in vitro* with increases in the ratio of a G_β antibody to $G_{\beta\gamma}$ (Murakami et al., 1992); the inhibitory potential of the G_β antibody is ranked as follows: RA >> SW = MS = KT, and the G_β RA antibody interferes with the interaction between the G_α and $G_{\beta\gamma}$ subunits. Activation of PI3K or immunoprecipitation of a p85 complex by the G_β RA antibody and disruption of this complex by PTX in the treated cells seem to imply that CL or aluminum fluoride treatment induces dissociation of the G_α and $G_{\beta\gamma}$ subunits of G proteins, including those sensitive to PTX, and immunoprecipitates the $G_{\beta\gamma}$ subunits dissociated from these G proteins, and also that complexes are not formed through the G_β subunit peptide that is recognized by the G_β RA antibody.

p85/p110α and p85/p110β PI3K isoforms are activated by a phosphotyrosyl peptide (that contains a YMXM motif that interacts with PI3K p85) or by $G_{\beta\gamma}$ or both *in vitro* (Hazeki et al., 1998). Rat adipocytes express both of these isoforms (Ozanne et al., 1997); we detected a p85 subunit-containing complex by immunoprecipitation with IRS-I (which has the YMXM motif) or G_β RA antibody. Previous work showed that 10-min adrenergic stimulation in rat adipocytes does not activate PI3K in immunoprecipitates with a phosphotyrosine antibody (Kelly et al., 1992). CL or aluminum fluoride treatment may regulate a PI3K p85/p110 isoform via $G_{\beta\gamma}$ in order to produce 3-phosphoinositides or activate PI3K in rat adipocytes.

The G_β-derived peptide recognized by the G_β RA antibody shows sequence similarity with the peptides of other types of G_β ($G_{\beta 1\text{-}5}$, Watson et al., 1994). The p85 subunit of PI3K forms a dimer with the p110 catalytic subunit of PI3K. CL and aluminum fluoride-formed complexes in immunoprecipitates with the G_β RA antibody seem to contain a type of G_β subunit and the p110 subunit of PI3K and may be useful for examining the machinery involved in the CL-induced PI3K responses associated with G_β. Rat adipocytes express not only the p85 subunit of PI3K but also, at a lower level, the p55 subunit of PI3K (Inukai et al., 1997) and are sensitive to an inhibitor of another type of PI3K (PI3Kγ) that is activated by $G_{\beta\gamma}$ (Baragli et al., 2011). We cannot exclude the possibility that other types of PI3K are involved in CL- and aluminum fluoride-induced responses in rat adipocytes.

Expression of a 3-phosphoinositide-responsive kinase in rat adipocytes activates an ERK isoform or PKB (activation that is induced by CL) (Grillo et al., 1999). CL may activate a PI3K activation-dependent molecular machinery via a $G_{\beta\gamma}$ type in order to activate the PKB or ERK pathway. The number of PM GLUT molecules in rat adipocytes is increased by expression of an active form of PI3K or PKB (Tanti et al., 1997) and by expression of the 3-phosphoinositide-responsive kinase (Standaert et al., 1997; Grillo et al., 1999). Treatment of rat adipocytes with ISO or the GTP analogue GTPγS, which activates both trimeric and small G proteins, induces increased PM GLUT molecules (Shirakura et al., 1990; Baldini et al., 1991) or PI3K activation (Standaert et al., 1998). CL-induced PI3K activation, CL-induced p85 complex formation, or aluminum fluoride-induced PTX-sensitive complex formation may be helpful for clarifying the machinery involved in the PKB or MAPK response induced by CL, induction of PM GLUT caused by β-AR activation, and GTPγS-regulated PI3K activation and subsequent response.

Glucose transport increases in rat white adipocytes exposed to ISO or CL treatment and also adrenergic stimulation for 15 or 30 min (Ludvigsen et al., 1980; Shirakura et al., 1990; Ohsaka et al., 1998) or exposed to an activator of G proteins (that include PTX-sensitive G proteins) for 10 or 30 min (Suzuki et al., 1992; Standaert et al., 1998); ISO or GTPγS treatment increases glucose transport together with PM GLUT translocation or after PI3K activation. The signalling mechanisms underlying these responses of increased glucose transport in rat white adipocytes are not well understood. CL and aluminum fluoride-induced responses related to PI3K may also be helpful for elucidating the mechanisms involved in glucose transport responses, which are increased by adrenoceptor regulation and by G-protein signalling activators in rat white adipocytes.

AMPK positively or negatively regulates lipolysis and adipose cell size. Activated MAPK modulates HSL *in vitro*. In L1 adipocytes, the activity of AMPKα1 is several-fold higher than that of AMPKα2 (Salt et al., 2000). An AMPK activator phosphorylates acetyl-CoA carboxylase (inhibits fatty acid synthesis activation) (Gormand et al., 2011) and induces PM GLUT translocation and glucose transport (Yamaguchi et al., 2005) in L1 adipocytes. A p85-containing immunocomplex activates PI3K and produces 3-phosphoinositides. Activated PI3K is induced by $G_{\beta\gamma}$ *in vitro* and induces PM GLUT

translocation in rat adipocytes. The kinase-related responses observed in this study may play a regulatory role in lipolytic and anti-lipolytic processes, lipogenic inhibition, G protein-associated signalling to PI3K and the subsequent responses, and glucose transport-related responses. Further analysis is needed to clarify the events that are related to the responses induced in this study and to determine the role of these responses.

Molecular targeting experiments, which are designed on the basis of similar responses to separate signals, make it possible to work toward elucidation of the machinery involved in receptor signalling. Inhibitory experiments targeting CL- and aluminum fluoride-responsive molecules are needed to determine the signalling machinery involved in adrenoceptor responses. We provide possible clues for clarifying the signalling machinery involved in adrenergic and β-AR responses, including those of β_3-AR, in mouse-derived adipocytes and rat white adipocytes. Our findings advance the understanding of adrenoceptor responses in adipose cells and of the cellular signalling machinery present in the cells.

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