

## SHORT COMMUNICATION

## CL316243 Induces Phosphatidylinositol 3,4,5-triphosphate Production in Rat Adipocytes in an Adenosine Deaminase-, Pertussis Toxin-, or Wortmannin-Sensitive Manner

Y. OHSAKA<sup>1,3</sup>, Y. NOMURA<sup>2,3</sup><sup>1</sup>Department of Pharmacology, Faculty of Pharmaceutical Sciences, Chiba Institute of Science, Choshi, Chiba, Japan, <sup>2</sup>Yokohama College of Pharmacy, Yokohama, Kanagawa, Japan,<sup>3</sup>Department of Pharmacology, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Hokkaido, Japan

Received August 5, 2015

Accepted February 12, 2016

On-line March 15, 2016

### Summary

The effect of  $\beta_3$ -adrenoceptor ( $\beta_3$ -AR) agonists on adipocytes treated or not treated with signaling modulators has not been sufficiently elucidated. Using rat epididymal adipocytes (adipocytes) labeled with [<sup>32</sup>P]orthophosphate, we found that treatment with the selective  $\beta_3$ -AR agonist CL316243 (CL; 1  $\mu$ M) induces phosphatidylinositol (PI) 3,4,5-triphosphate (PI[3,4,5]P<sub>3</sub>) production and that this response is inhibited by adenosine deaminase (ADA, an adenosine-degrading enzyme; 2 U/ml), pertussis toxin (PTX, an inactivator of inhibitory guanine-nucleotide-binding protein; 1  $\mu$ g/ml), or wortmannin (WT, a PI-kinase inhibitor; 3  $\mu$ M). The results showed that CL induced PI(3,4,5)P<sub>3</sub> production in intact adipocytes and that this production was affected by signaling modulators. Taken together, our findings indicate that CL produces PI(3,4,5)P<sub>3</sub> in an ADA-sensitive, PTX-sensitive, or WT-sensitive manner and will advance understanding of the effect of  $\beta_3$ -AR agonists on adipocytes.

### Key words

Adipocytes • CL316243 (disodium (*R,R*)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate) • Phosphatidylinositol 3,4,5-triphosphate

### Corresponding author

Y. Ohsaka, Chiba Institute of Science, 15-8 Shiomi-cho, Choshi, Chiba 288-0025, Japan. E-mail: y-ohsaka@live.jp; y-ohsaka@cis.ac.jp

Treatment of adipocytes with  $\beta_3$ -adrenoceptor ( $\beta_3$ -AR) agonists, including CL316243 (CL), induces various responses, including glucose transport and protein-kinase B phosphorylation (PKB activation) in a signaling modulator-sensitive manner (Ohsaka *et al.* 1998, Zmuda-Trzebiatowska *et al.* 2007). Polymorphic studies have indicated that signaling molecules associated with  $\beta_3$ -AR can be targeted to improve adipocyte dysregulation (Arner and Hoffstedt 1999). However, the effect of  $\beta_3$ -AR agonists on adipocytes, including those treated with signaling modulators, has not been fully elucidated.

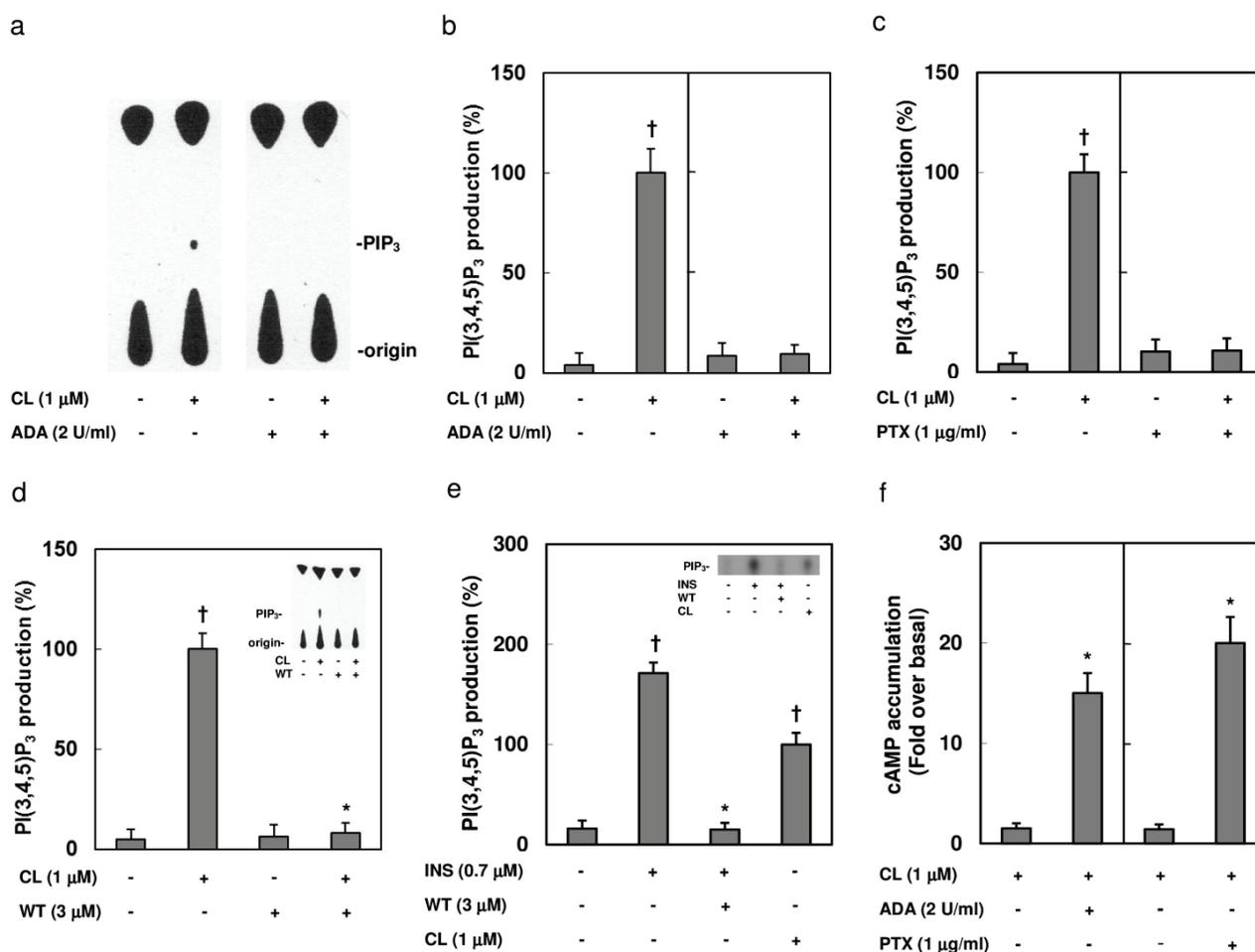
Phosphatidylinositol (PI) 3,4,5-triphosphate (PI[3,4,5]P<sub>3</sub>) is a component of the PI 3-kinase-related pathway and has been shown to activate PKB (Walker *et al.* 1998). Additionally, expression of a PI(3,4,5)P<sub>3</sub>-responsive kinase in rat epididymal adipocytes (adipocytes) induces an insulin (INS)-responsive glucose transport-related response (Standaert *et al.* 1997). PI 3-kinase activation is induced in adipocytes treated with CL as well as INS (Ohsaka *et al.* 2014). In a previous study, PI(3,4,5)P<sub>3</sub> was produced by INS treatment (0.1  $\mu$ M), which peaked at about 1 min, in adipocytes in a signaling modulator (e.g. wortmannin [WT])-sensitive manner (Takasuga *et al.* 1999). However, it is unclear whether CL treatment produces phosphoinositides in intact adipocytes. The effect of CL treatment for 1 min on PI(3,4,5)P<sub>3</sub> production is

unknown.

Adenosine is released from adipocytes and degraded in adenosine deaminase (ADA; 2 U/ml, <10 min)-treated adipocytes (Shirakura and Tokumitsu 1990). Adipocyte membranes treated with an adenosine-receptor agonist modulate the inhibitory guanine-nucleotide-binding (G) protein  $G_i$  which can be affected by pertussis toxin (PTX) (Mitchell *et al.* 1989, Soeder *et al.* 1999). In addition, WT treatment (from 0.1 to <10  $\mu$ M) inactivates the kinases that produce PI 3- and PI 4-monophosphates (Okada *et al.* 1994). Previous studies showed that adipocytes treated with ADA (2 U/ml, 30 min), PTX (0.2  $\mu$ g/ml, 180 min), or WT (0.1  $\mu$ M, 10 min) exhibit altered responses to  $\beta_3$ -AR agonists (0.01-100  $\mu$ M, 10-30 min) (Chaudhry *et al.*

1994, Ohsaka *et al.* 1997, 1998, Zmuda-Trzebiatowska *et al.* 2007), including ADA- or PTX-sensitive alteration of  $\beta_3$ -AR agonist-induced adenosine 3',5'-cyclic monophosphate (cAMP) accumulation. It is unclear whether CL produces PI(3,4,5) $P_3$  in an ADA-, PTX-, or WT-sensitive manner.

To investigate the effect of  $\beta_3$ -AR agonists on signaling modulator-treated or -untreated adipocytes, we examined whether treatment with CL (1  $\mu$ M; Lederle Laboratories, Wayne, NJ) for 1 min produces PI(3,4,5) $P_3$  and whether this response is affected by treatment with ADA (2 U/ml, 10 min; Sigma-Aldrich Co., St. Louis, MO), PTX (1  $\mu$ g/ml, 60 min; Kaken Pharmaceutical Co., Tokyo, Japan), or WT (3  $\mu$ M, 1 min; Kyowa Hakko Kogyo Co., Tokyo, Japan).



**Fig. 1.** Effect of CL alone or in combination with ADA, PTX or WT on PI(3,4,5) $P_3$  production in adipocytes. [ $^{32}$ P]orthophosphate-labeled adipocytes that were treated or not treated with ADA (2 U/ml, 10 min) or PTX (1  $\mu$ g/ml, 60 min) were incubated for 1 min with or without CL (1  $\mu$ M; **a-c**). In addition, [ $^{32}$ P]orthophosphate-labeled adipocytes treated with or without WT (3  $\mu$ M, 1 min) were incubated for 1 min with or without CL (**d** and **e**) or INS (0.7  $\mu$ M; **e**). Furthermore, adipocytes treated or not treated with ADA or PTX were incubated for 10 min with or without CL (**f**). The radioactivity of PI(3,4,5) $P_3$  (PIP $_3$ ) that was separated by TLC and the accumulation of cAMP in adipocytes were determined. Values are presented as means  $\pm$  standard deviation of 3 or 4 experiments. Autoradiographic images represent typical results. The results were analyzed using analysis of variance with Scheffe's test or unpaired Student's *t*-test. †  $P < 0.05$  vs. cells treated without agents (**b-e**). \*  $P < 0.05$  vs. cells treated with CL alone (**d** and **f**) or INS (**e**).

Adipocytes ( $10^6$  cells/ml) were prepared from rat epididymal adipose tissues as described previously (Ohsaka *et al.* 2014). Animal experiments, which were approved by the institutional review board, were conducted in accordance with the guidelines established by the Japanese Association for Laboratory Animal Science (JALAS) (JALAS 1987). The adipocytes were incubated for 2 h in phosphate-free Krebs-Ringer bicarbonate buffer containing 3 % bovine serum albumin (Sigma-Aldrich Co.) in the presence of carrier-free [ $^{32}$ P]orthophosphate (DuPont NEN, Boston, MA) at 37 °C. [ $^{32}$ P]-labeled adipocytes treated with or without agents were immediately separated from the medium by centrifugation at 500 x g for 20 s, and phospholipids were extracted and separated by thin-layer chromatography (TLC) in a solution of chloroform/acetone/methanol/acetic acid/water (80:30:26:24:14, v/v) as described previously (Arcaro and Wymann 1993). The radioactivity of PI(3,4,5)P<sub>3</sub> was detected and quantified using a Fuji BAS2000 Bioimaging Analyzer (Fuji Photo Film Co., Tokyo, Japan).

To confirm the function of isolated adipocytes, we examined whether treatment with INS (0.7 μM, 1 min; Sigma-Aldrich Co.) produces PI(3,4,5)P<sub>3</sub> in a WT-sensitive manner and whether ADA or PTX treatment alters CL (1 μM, 10 min)-induced cAMP accumulation; cAMP accumulation was determined as described previously (Shirakura and Tokumitsu 1990). Treatment of the adipocytes with INS induced WT-sensitive PI(3,4,5)P<sub>3</sub> production (Fig. 1e), and ADA or PTX treatment altered CL-induced cAMP accumulation (Fig. 1f).

PI(3,4,5)P<sub>3</sub> is produced from PI 4,5-bisphosphate (PI[4,5]P<sub>2</sub>) in a PI 3-kinase p85 regulatory subunit-containing immunocomplex (Kelly and Ruderman 1993). In G-protein subunit β (Gβ) antibody immunoprecipitates, CL treatment increases the PI 3-kinase p85 subunit level and phosphorylates the 3'-position of the inositol ring (Ohsaka *et al.* 2014). In addition, PI(3,4,5)P<sub>3</sub> is dephosphorylated by a PI(3,4,5)P<sub>3</sub> phosphatase *in vitro*, and this dephosphorylation is enhanced by PI(4,5)P<sub>2</sub> (Campbell *et al.* 2003). As shown in Figure 1a-e, CL treatment produced PI(3,4,5)P<sub>3</sub>. This response is presumed to be regulated by a PI or phospho-PI kinase(s) and/or a phospho-PI phosphatase(s). Production of PI(3,4,5)P<sub>3</sub>, which is produced by INS, was induced in CL-treated intact adipocytes.

PI(3,4,5)P<sub>3</sub> is able to activate PKB isoforms (Walker *et al.* 1998), including PKB-α/-β (which can be

phosphorylated). Additionally, treatment of adipocytes with PI(3,4,5)P<sub>3</sub> activates the protein-kinase C (PKC) isoform PKC-ζ (Standaert *et al.* 1997). The number of glucose transporters (GLUT4) in the plasma membrane (PM) is increased by expression of PKB-α (Tanti *et al.* 1997) or PKC-ζ (Standaert *et al.* 1997). CL-induced PI(3,4,5)P<sub>3</sub> production may regulate activation of PKB-α and/or -β the expression of which is observed in adipocytes (Walker *et al.* 1998) and PKC-ζ and may induce PM GLUT4 expression.

Adipocytes have the G<sub>i</sub>-coupled A1 adenosine-receptor (Burnstock 2014). The G-protein subunit Gα of G<sub>i1</sub> or G<sub>i2</sub> is modified by PTX (Mitchell *et al.* 1989); PTX inhibits receptor signaling-induced G-protein dissociation of the Gα and Gβγ subunits (Katada *et al.* 1984). ADA or PTX treatment did not induce CL-induced PI(3,4,5)P<sub>3</sub> production (Fig. 1a-c). The CL-induced PI(3,4,5)P<sub>3</sub> production may be regulated by adenosine-sensitive molecules including the A1 adenosine-receptor and by a PTX-sensitive G<sub>i</sub> isoform(s). Adipocytes express the PI 3-kinase isoforms p85/p110-α/ p110-β (Ohsaka *et al.* 2014 and Discussion section therein) and PI 4-kinase (Okada *et al.* 1994). The p85/p110-β isoform is activated by Gβγ *in vitro* (Hazeki *et al.* 1998). WT inactivates these kinases. In Figure 1d, CL-induced PI(3,4,5)P<sub>3</sub> production was inhibited by WT; such an inhibitory effect was not observed when another response (lipolysis) was measured (data not shown). Adipocyte membranes treated with β<sub>3</sub>-AR agonists modulate G proteins (Soeder *et al.* 1999), including G<sub>i</sub> which can regulate p85 complex formation in Gβ antibody immunoprecipitates (Ohsaka *et al.* 2014). CL-induced PI(3,4,5)P<sub>3</sub> production may be regulated by WT-sensitive PI kinases, including a PI 3-kinase isoform of p85/p110.

In this study, CL induced the production of PI(3,4,5)P<sub>3</sub> in intact adipocytes, and this production was affected by signaling modulators. Our findings indicate that CL produces PI(3,4,5)P<sub>3</sub> in an ADA-sensitive, PTX-sensitive, or WT-sensitive manner and will advance understanding of the effect of β<sub>3</sub>-AR agonists on adipocytes.

### Conflict of Interest

There is no conflict of interest.

### Acknowledgements

We thank Dr. Y. Tokumitsu (Hokkaido University, Japan) for helpful advice on the measurement of cAMP.

## References

- ARCARO A, WYMAN MP: Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem J* **296**: 297-301, 1993.
- ARNER P, HOFFSTEDT J: Adrenoceptor genes in human obesity. *J Intern Med* **245**: 667-672, 1999.
- BURNSTOCK G: Purinergic signalling in endocrine organs. *Purinergic Signal* **10**: 189-231, 2014.
- CAMPBELL RB, LIU F, ROSS AH: Allosteric activation of PTEN phosphatase by phosphatidylinositol 4,5-bisphosphate. *J Biol Chem* **278**: 33617-33620, 2003.
- CHAUDHRY A, MACKENZIE RG, GEORGIC LM, GRANNEMAN JG: Differential interaction of  $\beta_1$ - and  $\beta_3$ -adrenergic receptors with  $G_i$  in rat adipocytes. *Cell Signal* **6**: 457-465, 1994.
- HAZEKI O, OKADA T, KUROSU H, TAKASUGA S, SUZUKI T, KATADA T: Activation of PI 3-kinase by G protein  $\beta\gamma$  subunits. *Life Sci* **62**: 1555-1559, 1998.
- JALAS: Guidelines on animal experimentation (in Japanese). *Exp Anim* **36**: 285-288, 1987.
- KATADA T, NORTHUP JK, BOKOCH GM, UI M, GILMAN AG: The inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. Subunit dissociation and guanine nucleotide-dependent hormonal inhibition. *J Biol Chem* **259**: 3578-3585, 1984.
- KELLY KL, RUDERMAN NB: Insulin-stimulated phosphatidylinositol 3-kinase. *J Biol Chem* **268**: 4391-4398, 1993.
- MITCHELL FM, GRIFFITHS SL, SAGGERSON ED, HOUSLAY MD, KNOWLER JT, MILLIGAN G: Guanine-nucleotide-binding proteins expressed in rat white adipose tissue. Identification of both mRNAs and proteins corresponding to  $G_{i1}$ ,  $G_{i2}$  and  $G_{i3}$ . *Biochem J* **262**: 403-408, 1989.
- OHSAKA Y, TOKUMITSU Y, NOMURA Y: Suppression of insulin-stimulated phosphatidylinositol 3-kinase activity by the  $\beta_3$ -adrenoceptor agonist CL316243 in rat adipocytes. *FEBS Lett* **402**: 246-250, 1997.
- OHSAKA Y, MURAKAMI T, YOSHIDA T, TOKUMITSU Y: Comparison of atypical  $\beta_3$ -adrenoceptor agonists with their respective metabolic activities in rat white adipocytes. *Jpn J Pharmacol* **77**: 41-51, 1998.
- OHSAKA Y, NISHINO H, NOMURA Y: 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. *Folia Biol (Praha)* **60**: 168-179, 2014.
- OKADA T, KAWANO Y, SAKAKIBARA T, HAZEKI O, UI M: Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. *J Biol Chem* **269**: 3568-3573, 1994.
- SHIRAKURA S, TOKUMITSU Y: Insulin-stimulated glucose transport regulated by adenylyl cyclase system in rat adipocytes. *Comp Biochem Physiol* **96A**: 503-509, 1990.
- SOEDER KJ, SNEDDEN SK, CAO W, DELLA ROCCA GJ, DANIEL KW, LUTTRELL LM, COLLINS S: The  $\beta_3$ -adrenergic receptor activates mitogen-activated protein kinase in adipocytes through a  $G_i$ -dependent mechanism. *J Biol Chem* **274**: 12017-12022, 1999.
- STANDAERT ML, GALLOWAY L, KARNAM P, BANDYOPADHYAY G, MOSCAT J, FARESE RV: Protein kinase C- $\zeta$  as a downstream effector of phosphatidylinositol 3-kinase during insulin stimulation in rat adipocytes. *J Biol Chem* **272**: 30075-30082, 1997.
- TAKASUGA S, KATADA T, UI M, HAZEKI O: Enhancement by adenosine of insulin-induced activation of phosphoinositide 3-kinase and protein kinase B in rat adipocytes. *J Biol Chem* **274**: 19545-19550, 1999.
- TANTI J-F, GRILLO S, GRÉMEAUX T, COFFER PJ, VAN OBERGHEN E, LE MARCHAND-BRUSTEL Y: Potential role of protein kinase B in glucose transporter 4 translocation in adipocytes. *Endocrinology* **138**: 2005-2010, 1997.
- WALKER KS, DEAK M, PATERSON A, HUDSON K, COHEN P, ALESSI DR: Activation of protein kinase B  $\beta$  and  $\gamma$  isoforms by insulin *in vivo* and by 3-phosphoinositide-dependent protein kinase-1 *in vitro*: comparison with protein kinase B  $\alpha$ . *Biochem J* **331**: 299-308, 1998.
- ZMUDA-TRZEBIATOWSKA E, MANGANIELLO V, DEGERMAN E: Novel mechanisms of the regulation of protein kinase B in adipocytes; implications for protein kinase A, Epac, phosphodiesterases 3 and 4. *Cell Signal* **19**: 81-86, 2007.