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Suppression of insulin-stimulated phosphatidylinositol 3-kinase activity by the β_3-adrenoceptor agonist CL316243 in rat adipocytes

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Abstract Insulin increased 2-deoxyglucose (2-DG) uptake via the translocation of glucose transporter (GLUT) 4 to the plasma membrane fraction in rat adipocytes. The stimulatory actions of insulin were accompanied by both an increase in the immunoreactive p85 subunit of phosphatidylinositol (PI) 3-kinase in the plasma membrane fractions and PI 3-kinase activation by tyrosine phosphorylation of the p85 subunit. The β_3-adrenoceptor agonist CL316243 (CL) suppressed all the insulin actions in adenosine deaminase (ADA)-treated cells, but was without effect in non-ADA-treated cells. The inhibitory effects of CL on GLUT 4 translocation and PI 3-kinase activation were abolished by the addition of N^6-phenylisopropyl adenosine. Cholera toxin treatment, which markedly increased intracellular cAMP levels, suppressed increases in the levels of GLUT 4 and PI 3-kinase in the plasma membrane fractions in response to insulin. In addition, dibutyryl (Bt_2) cAMP also impaired the activation of PI 3-kinase by insulin. These results indicated that CL suppressed insulin-stimulated glucose transport under conditions where cAMP levels were markedly increased (~12-fold). The inhibitory actions of PI 3-kinase activation by insulin were exerted even when cAMP, 8-bromo-cAMP, or Bt_2 cAMP was added to immunoprecipitates of the p85 subunit of PI 3-kinase, after treating the cells with insulin. These results suggest that CL suppressed insulin-stimulated PI 3-kinase activity via a cAMP-dependent mechanism, at least in part, direct cAMP action in ADA-treated adipocytes, by which PI 3-kinase activation was inhibited, resulting in the decrease in GLUT 4 translocation and subsequent 2-DG uptake in response to insulin.

Key words: CL316243; Phosphatidylinositol 3-kinase; 2-Deoxyglucose uptake; Glucose transporter 4; Insulin; Rat adipocyte

1. Introduction

The tyrosine kinase of the β-subunits of the insulin receptor activated by insulin phosphorylates the tyrosine residues of insulin receptor substrate (IRS) 1, leading to the translocation of glucose transporter (GLUT) 4 to the plasma membranes and subsequent glucose transport [1,2]. It has been reported that glucagon [3], VIP [4], or catecholamines [5], which increase adenylyl cyclase activity, suppress insulin-stimulated glucose transport, suggesting that the inhibitory actions are due to a decrease in insulin binding [6–8], insulin receptor tyrosine kinase activity [9,10], or the intrinsic activity of the glucose transporter by cAMP-dependent mechanisms [11–13]. It is, however, unclear by which mechanism cAMP impairs some intermediary pathway between those events. We demonstrated that epinephrine, isoproterenol, glucagon, and ACTH were able to stimulate glucose uptake under normal incubation conditions where cAMP levels were increased up to ~3-fold [14].

Recently, it was demonstrated that phosphatidylinositol (PI) 3-kinase is involved in insulin-regulated movement of GLUT 4 to the plasma membranes in rat adipocytes [15,16] or 3T3-L1 adipocytes [17]. Therefore, insulin receptor activation caused the rapid delivery of IRS-1/PI 3-kinase complexes to intracellular membranes where 3′-phosphoinositides had accumulated and functioned in the membrane-associated events which accompanied the translocation of GLUT 4 to the plasma membrane. On the other hand, adrenoceptors (ARs) of the rat adipocytes are mainly β_1 and β_3 types according to pharmacological analysis [18,19]. Hence, it is possible that β_3-AR agonists affect insulin-stimulated glucose transport via PI 3-kinase activity. We investigated the role(s) of typical β_3-ARs on PI 3-kinase activity and GLUT 4 translocation related to insulin-stimulated glucose transport using a selective β_3-agonist, CL316243 (CL) [20].

2. Materials and methods

2.1. Materials

The β_3-AR agonist CL and the cAMP assay kit were generously provided by Lederle Laboratories (USA) and by Yamasa Shoyu Co. (Japan), respectively. Anti-rabbit GLUT 4 polyclonal antibody (C-terminal polypeptide) was a kind gift from Dr. O. Ezaki (National Institute of Nutrition, Japan). Collagenase type I was purchased from Worthington Biochemical Corp. (USA). 2-Deoxy-D-1,2-[^{3}H]glucose ([^{3}H]2-DG) and horseradish peroxidase-linked anti-mouse IgG were from Amersham (Japan). Anti-mouse phosphotyrosine monoclonal antibodies and anti-rabbit p85 subunit of PI 3-kinase polyclonal antibodies for both immunoblotting and immunoprecipitation were from Zymed Laboratory, Inc. (USA) and from Upstate Biotechnology, Inc. (USA), respectively. Cholera toxin (CTX) and phosphatidylinositol were from List Biological Laboratories, Inc. (USA) and Serdary Research Laboratories (USA), respectively. The enhanced chemiluminescence system (ECL Western blotting detection kit) and protein G-Sepharose 4 Fast Flow were from Amersham (USA) and Pharmacia (USA), respectively. Insulin, leupeptin, aprotinin, Ro1724 (phosphodiesterase inhibitor), adenosine deaminase (ADA), Nonidet (N) P-40, 2-DG, phenylisopropyladenosine (PIA), cAMP, cGMP, AMP, GTP, bovine serum albumin (BSA, FFA free), 8-bromo-AMP, and phenylmethylsulfonylfluoride (PMSF) were all from Sigma Chemical Co. (USA). 8-Bromo-cAMP and dibutyryl (Bt_2) cAMP were from Yamasa Shoyu Co. (Japan).

2.2. Preparation of adipocytes

White adipocytes were isolated from the epididymal adipose tissues of male Wistar rats weighing 200–300 g by the collagenase digestion method as described elsewhere [21].

2.3. Measurement of glucose transport activity

Isolated adipocytes were preincubated with various agents and further incubated for 3 min with 0.2 mM [^{3}H]2-DG in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 3% BSA under an atmosphere of 95% O_2–5% CO_2. 2-DG uptake was measured as described previously [21].

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2.4. Measurement of cAMP accumulation

Isolated adipocytes were incubated with various agents for 10 min in Krebs-Ringer-HEPES buffer (pH 7.4) containing 3% BSA and 0.3 mM Ro1724. cAMP accumulation was measured by radioimmunoassay as described previously [22].

2.5. The measurement of translocation of GLUT 4 and PI 3-kinase to plasma membrane fractions

Isolated adipocytes were incubated with various agents. The cells were immediately separated from the medium by centrifugation. Cells were washed with buffer A (20 mM HEPES, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM PMSF, 5 μg/ml leupeptin, and 10 μg/ml aprotinin) and suspended in buffer A. Cell suspensions were homogenized in a Dounce homogenizer and centrifuged for 5 min at 3000×*g*. The supernatant was centrifuged for 20 min at 15000×*g*. Plasma membrane fractions were used for immunological detection of GLUT 4 proteins and p85 of PI 3-kinase.

2.6. Immunoprecipitation and/or Western blot analysis

Plasma membrane fractions prepared as described above were lysed in lysis buffer B (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1 mM Na_3VO_4, 0.25% Na-deoxycholate, 1 mM PMSF, 10 mM NaF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Lysates were immunoprecipitated with p85 of PI 3-kinase antibody preadsorbed on protein G-Sepharose beads. Lysates or immune complexes were subjected to 10% SDS-PAGE, after which the proteins were transferred to Immobilon-P filters. The filters were incubated with the appropriate antibody. Blots were developed using the ECL system employing horseradish peroxidase-linked anti-mouse IgG as described elsewhere [23,24].

2.7. Measurement of PI 3-kinase activity using immunoprecipitates for p85 antibody

Isolated adipocytes were incubated with various agents. The cells were separated from the medium by centrifugation and lysed in chilled buffer (pH 7.4) containing 0.25 M sucrose, 50 mM HEPES, 150 mM NaCl, 1% NP-40, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 2 mM Na_3VO_4, and 0.5 mM PMSF. After centrifugation at 15000×*g* for 20 min, the supernatants were immunoprecipitated with p85 antibody, followed by protein G-Sepharose. The immunoprecipitates were used for the PI 3-kinase assay as described elsewhere [25].

The quantitativeness of the relative intensities on autoradiograms was analyzed on a Macintosh computer using the NIH Image program (developed at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, VA, part number PB95-500195GEI).

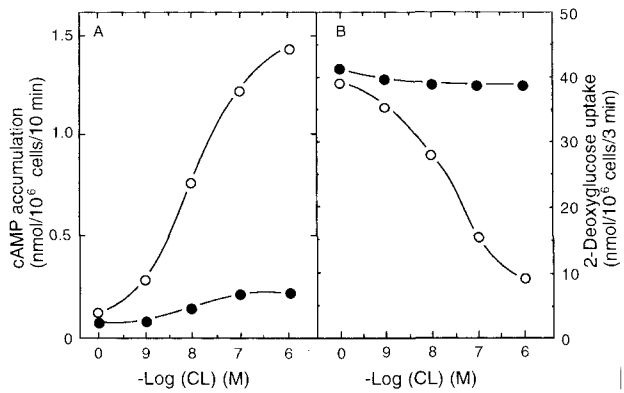

Fig. 1. Effects of CL on cAMP accumulation and on insulin-stimulated 2-DG uptake. Adipocytes were preincubated for 30 min in the presence (○) or absence (●) of ADA (2 U/ml). A: Each cell suspension was incubated with the indicated concentrations of CL for 10 min. The amount of cAMP accumulation was determined. B: Each cell suspension was further preincubated with the indicated concentrations of CL in combination with insulin (70 nM) for 30 min (20 min in the case of insulin) and incubated with 0.2 mM [^{3}H]2-DG. 2-DG uptake was determined.

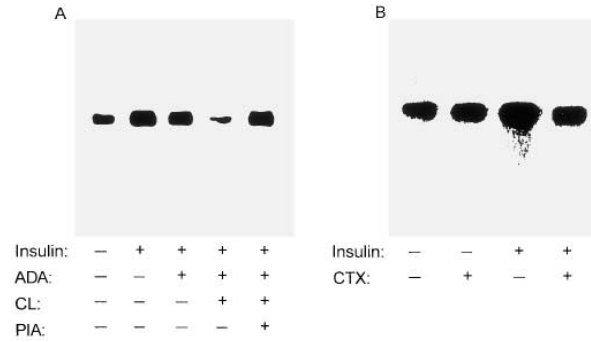

Fig. 2. Effects of CL and CTX on insulin-stimulated GLUT 4 translocation. Adipocytes were preincubated for 30 min in the presence or absence of ADA (2 U/ml) (A) or CTX (10 μg/ml) (B). Each cell suspension was incubated with various agonists for 20 min. Plasma membrane fractions were prepared and GLUT 4 proteins were detected by immunoblotting with GLUT 4 antibodies. Insulin (70 nM); CL (0.1 μM); PIA (10 μM).

2.8. Protein assay

Protein was assayed by Bradford's method [26].

3. Results

3.1. Effects of CL and CTX on cAMP accumulation and insulin-stimulated 2-DG uptake

The effects of the β_3-agonist CL on cAMP accumulation and insulin-stimulated 2-DG uptake were examined in ADA-treated and non-treated cells (Fig. 1A,B). CL slightly increased cAMP levels (~3-fold), but did not affect insulin-stimulated 2-DG uptake in non-ADA-treated cells. In the ADA-treated cells, CL markedly increased cAMP levels (~12-fold) and suppressed insulin-stimulated 2-DG uptake dose-dependently. A rapid appearance of GLUT 4 in the plasma membranes in response to insulin occurred, leading to the increase in glucose uptake [27,28]. Hence, the effects of CL on GLUT 4 levels increased by insulin were examined by immunoblotting the GLUT 4 protein in the plasma membrane fractions (Fig. 2). Insulin increased the level of GLUT 4 protein in the plasma membrane fractions and the increase was completely abolished by CL in the ADA-treated cells. ADA treatment itself hardly attenuated the actions of insulin. The inhibitory action by CL was abolished by the addition of

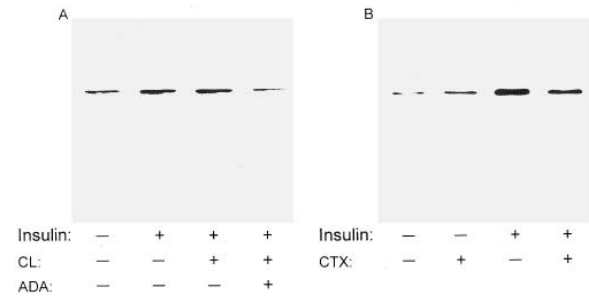

Fig. 3. Effects of CL and CTX on the p85 subunit of PI 3-kinase in the plasma membrane fractions. Adipocytes were preincubated for 30 min in the presence or absence of ADA (2 U/ml) (A) or CTX (10 μg/ml) (B). Each cell suspension was incubated with various agonists for 20 min. Plasma membrane fractions were prepared and p85 proteins were detected by immunoblotting with p85 subunit antibodies. Insulin (70 nM); CL (0.1 μM).

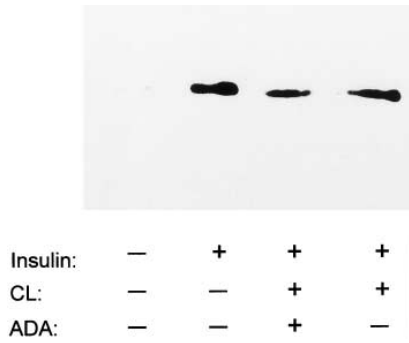

Fig. 4. Effects of CL on insulin-stimulated tyrosine phosphorylation of the p85 subunit of PI 3-kinase. Adipocytes were preincubated for 30 min in the presence or absence of ADA (2 U/ml). Each cell suspension was incubated with insulin (70 nM) with or without CL (0.1 μM) for 20 min. Cell lysates were subjected to immunoprecipitation with the p85 subunit antibody and analyzed by SDS-PAGE. Phosphotyrosine-containing proteins were detected by immunoblotting with a phosphotyrosine antibody.

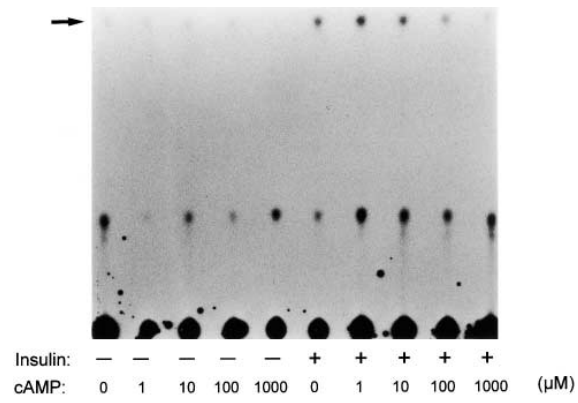

Fig. 6. Effects of cAMP on PI 3-kinase activity from insulin-stimulated adipocytes. Adipocytes were incubated with or without insulin (70 nM). Thereafter, PI 3-kinase activities were determined in the presence of the indicated concentrations of cAMP using immunoprecipitates for the p85 subunit.

the non-hydrolyzable adenosine analogue PIA (Fig. 2A). CTX [29], which caused a marked increase in cAMP levels via the ADP-ribosylation of Gsα, also impaired insulin-induced increases in GLUT 4 levels. The basal level of GLUT 4 was not affected by CTX treatment (Fig. 2B). These results indicate that CL suppressed insulin-stimulated glucose transport via the inhibition of GLUT 4 translocation induced by a marked increase in the cAMP levels with ADA or CTX treatment.

3.2. *Effects of CL and CTX on PI 3-kinase*

The effects of CL and CTX on the amount of PI 3-kinase were examined by immunoblotting p85 protein in the plasma membrane fractions (Fig. 3). Insulin caused an increase in p85 proteins and the increase was not affected by CL. In ADA-treated cells, CL abolished the increase in the p85 protein induced by insulin (Fig. 3A). CTX treatment also attenuated the increase in the p85 protein, but did not bring about a significant change in the basal p85 level (Fig. 3B). Effects of CL on insulin-stimulated tyrosine phosphorylation of the p85 were examined by immunoblotting with an anti-p85 antibody and an anti-phosphotyrosine antibody (Fig. 4). Insulin caused tyrosine phosphorylation of p85 and CL was without any effect on tyrosine phosphorylation. In contrast, in ADA-treated cells, CL greatly inhibited the tyrosine phosphorylation induced by insulin.

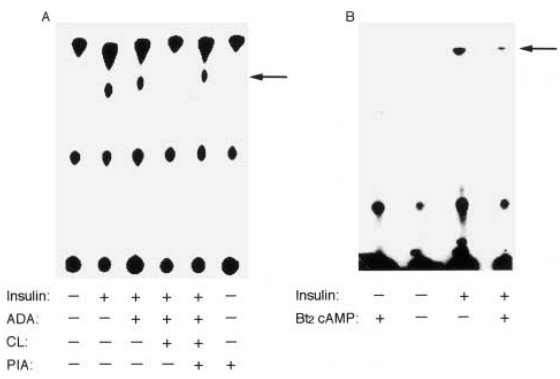

Fig. 5. Effects of CL and Bt_2 cAMP on insulin-stimulated PI 3-kinase activity. Adipocytes were preincubated for 30 min in the presence or absence of ADA (2 U/ml). Each cell suspension was incubated with or without insulin (70 nM) with or without CL (0.1 μM), PIA (10 μM) (A), or Bt_2 cAMP (0.2 mM) (B). PI 3-kinase activities were determined using immunoprecipitates of the p85 subunit.

3.3. *Effects of CL and Bt_2 cAMP on insulin-stimulated PI 3-kinase activity*

To test whether the inhibitory effect of CL on insulin-stimulated tyrosine phosphorylation of p85 leads to the suppression of PI 3-kinase activation, PI 3-kinase activity was assessed using the immunoprecipitates of p85 (Fig. 5A). Insulin greatly activated PI 3-kinase. CL inhibited insulin-stimulated PI 3-kinase in ADA-treated cells. The inhibitory action of CL was abolished by the addition of PIA. Neither PIA nor ADA treatment affected basal and insulin-stimulated PI 3-kinase activities. Bt_2 cAMP also suppressed insulin-stimulated PI 3-kinase activity and had little effect on the basal PI 3-kinase activity (Fig. 5B). These findings suggest that a marked increase in the cAMP level suppressed insulin-stimulated PI 3-

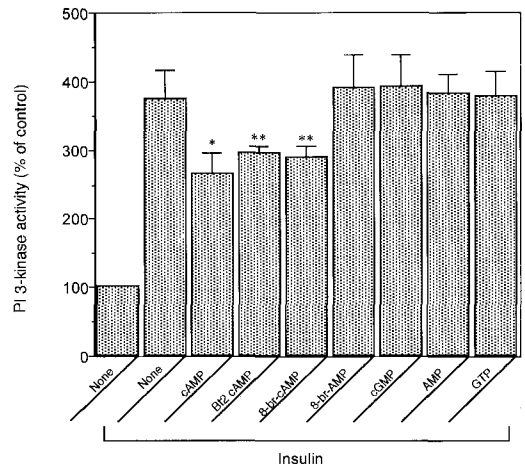

Fig. 7. Effects of various nucleotides on PI 3-kinase activity from insulin-stimulated adipocytes. Adipocytes were incubated with or without insulin (70 nM). Thereafter, PI 3-kinase activities were determined in the presence of various nucleotides at 100 μM using immunoprecipitates for the p85 subunit. Data represent means ± S.D. of four experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with insulin.

kinase activity, resulting in the suppression of GLUT 4 translocation and subsequent 2-DG uptake.

3.4. The inhibitory effect of cAMP on PI 3-kinase activity

To determine the mechanism responsible for the inhibition of insulin-induced PI 3-kinase activation by Bt_2 cAMP, cAMP was directly added to the immunoprecipitates of p85 subunit activated by insulin, and PI 3-kinase activity was assessed. cAMP had little effect on basal PI 3-kinase activity and inhibited insulin-stimulated PI 3-kinase activity in a concentration-dependent manner (Fig. 6). Direct effects of other nucleotides on insulin-stimulated PI 3-kinase were also observed. Addition of Bt_2 cAMP or 8-bromo-cAMP suppressed the insulin-stimulated PI 3-kinase similar to cAMP, whereas 8-bromo-AMP, cGMP, AMP, and GTP had no effect (Fig. 7).

4. Discussion

In rat adipocytes, the β_3-AR agonist CL caused a slight increase ($\sim$3-fold) in cAMP levels with no ADA treatment, but was without effect on insulin-stimulated 2-DG uptake. In contrast, CL caused marked cAMP accumulation ($\sim$12-fold) with ADA treatment and suppressed insulin-stimulated 2-DG uptake. These results indicate that the suppression of insulin-stimulated 2-DG uptake by CL is dependent on the elimination of adenosine in the incubation medium. Insulin stimulates glucose transport by translocation of mainly GLUT 4, but also, to a lesser extent, GLUT 1 [30], from the low density microsomes to the plasma membranes [27,28]. We also observed similar results (data not shown). Western blotting experiments with an antibody for GLUT 4 showed that the increase in the GLUT 4 protein in the plasma membrane fractions in response to insulin was not affected by CL in the absence of ADA and was abolished by CL in the presence of ADA, but was restored by further additions of PIA. CTX treatment, which induced a marked increase in cAMP levels in the absence of ADA, also suppressed the increase in GLUT 4 protein in the plasma membrane fractions induced by insulin. These results indicate that marked cAMP accumulation inhibits GLUT 4 translocation to the plasma membranes induced by insulin.

Insulin activates PI 3-kinase. This activation is needed to provoke GLUT 4-mediated glucose transport by insulin [25]. We showed that insulin-stimulated PI 3-kinase activities measured in the immunoprecipitates with p85 antibodies were inhibited by CL and the inhibition was abolished by further additions of PIA in the presence of ADA. Binding of tyrosyl-phosphorylated IRS-1 by insulin receptor tyrosine kinase to the p85 subunit of PI 3-kinase in response to insulin occurs and leads to the phosphorylation of the subunit [25]. Therefore, insulin stimulation is accompanied by translocation of the enzyme to the plasma membranes and tyrosine phosphorylation of the p85 protein [15,17]. In fact, Western blotting experiments with an antibody for p85 showed that insulin increased the amount of p85 protein in the plasma membrane fractions, and CL in the presence of ADA and CTX treatment suppressed this increase in the p85 protein. Furthermore, insulin increased an immunoreactive tyrosine-phosphorylated p85 protein, and the increase in the phospho-p85 protein was suppressed by CL in the presence of ADA. Heller-Harrison et al. [31] demonstrated that insulin directs the association of PI 3-kinase with GLUT 4-containing vesicles in rat adipocytes. Our results suggest that the inhibitory effect of CL on insulin-stimulated 2-DG uptake is mediated via cAMP-dependent mechanisms by which the appearance of PI 3-kinase on the cell surface, tyrosine-phosphorylation of p85, and subsequent GLUT 4 translocation to the plasma membrane fractions were inhibited.

On the other hand, it was shown that high concentrations of cAMP, 8-bromo-cAMP, and Bt_2 cAMP, but not other nucleotides directly inhibited PI 3-kinase activity in immunoprecipitates of p85 prepared from insulin-stimulated cells. Recently, it was reported that Bt_2 cAMP directly binds to GLUT 4, resulting in the inhibition of glucose transport [13]. It raises the possibility that cAMP has, at least in part, a direct effect on PI 3-kinase besides the cAMP-dependent mechanism involved in insulin-regulated movement of PI 3-kinase to the plasma membrane. It would be interesting to study the mechanism of a decrease in PI 3-kinase activity via the direct action(s) of cyclic nucleotides in the signaling pathway(s) of insulin-stimulated glucose transport.

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