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European Journal of Pharmacology 404 (2000) 63–68

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The stimulation of β_3-adrenoceptor causes phosphorylation of extracellular signal-regulated kinases 1 and 2 through a G_s- but not G_i-dependent pathway in 3T3-L1 adipocytes

Katsushige Mizuno, Yasunari Kanda, Yasutomi Kuroki, Yasuhiro Watanabe *

Department of Pharmacology, National Defense Medical College, 3-2 Namiki, Tokorozawa 359-8513, Japan

Received 27 April 2000; received in revised form 3 August 2000; accepted 8 August 2000

Abstract

The treatment of 3T3-L1 adipocytes with three β_3-adrenoceptor agonists, (±)-(R^*,R^*)-(4-[2-([2-(3-chlorophenyl)-2-hydroxyethyl]amino)propyl]phenoxy)acetic acid (BRL37344), 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1, 3-dihydro-2H-benzimidazol-2-one (CGP12177) and [(7S)7-{(2R)2-(3-chlorophenyl)-2-hydroxyethyl-amino}-5,6,7,8-tetrahydronapht-2-yl]ethyl oxyacetate, hydrochloride (SR58611) induces phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2). The phosphorylations were not affected by pretreatment of the adipocytes with pertussis toxin, whereas the same treatment completely abolished lisophosphatidic acid-induced phosphorylation of ERK1/2, suggesting the role of pertussis toxin-insensitive G protein in the ERK1/2 phosphorylation by stimulation with the β_3-adrenoceptor agonists. The phosphorylation of ERK1/2 was mimicked by treating the adipocytes with cholera toxin, a direct activator of stimulatory G (G_s) protein. In addition, the ERK1/2 phosphorylations by the β_3-adrenoceptor agonists were completely diminished by long-term treatment of the adipocytes with cholera toxin (100 ng/ml, 24 h), whereas that obtained with lisophosphatidic acid stimulation was not. Our findings strongly suggest that the three β_3-adrenoceptor agonists induce ERK1/2 phosphorylation in 3T3-L1 adipocytes through a G_s protein-dependent cascade.

Keywords: MAP (mitogen-activated protein) kinase cascade; Regulation; Protein kinase, cAMP-dependent; Signal transduction

1. Introduction

β_3-Adrenoceptors are predominantly expressed in white and brown adipocytes, and mediate a number of diverse metabolic effects such as lipolysis (Van Liefde et al., 1992), increase of energy expenditure (Weyer et al., 1998) and reduction of food intake (Mantzoros et al., 1996). These effects have been shown to disappear in β_3-adrenoceptor-knockout mice (Susulic et al., 1995). On the other hand, re-expression of the receptors in adipocytes recovered these effects (Grujic et al., 1997), demonstrating the importance of β_3-adrenoceptor-mediated responses in functioning adipocytes. Long-term treatment of obese and type 2 diabetic rodents with β_3-adrenoceptor agonists reduces fat stores and improves insulin action, with these effects also found in humans (Weyer et al., 1998). Thus, β_3-adrenoceptor agonists are expected to act as anti-obesity and/or anti-diabetic compounds (Sum et al., 1999). At the same time, the stimulation of β_3-adrenoceptor has been reported to inhibit insulin signaling such as glucose uptake (Klein et al., 1999), suggesting the need for further investigation of the signaling cascades of β_3-adrenoceptors in order to realize an efficient treatment for obesity and/or insulin resistance.

Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are activated by various extracellular signals and play critical roles in many physiological events such as proliferation, differentiation, homeostasis and survival (for review, see English et al., 1999). Recently, several studies have shown that the stimulation of β_3-adrenoceptor induced ERK1/2 phosphorylation, though the involved signaling pathways in the phenomena were controversial. In one of these studies, we showed that ERK1/2 phosphorylations by β_3-adrenoceptor agonists were sensitive to *N*-[2-(*p*-bromo-cinnamylamino)ethyl]-5-isoquinoline sulfonamide

* Corresponding author. Tel.: +81-42-1484; fax: +81-42-996-5191.
E-mail address: ndmcphar@sa2.so-net.ne.jp (Y. Watanabe).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00601-4

dihydrochloride (H89), the highly selective inhibitor of cAMP-dependent protein kinase, suggesting the participation of stimulatory G (G_s) protein and its signaling pathway (Mizuno et al., 1999). The other studies have shown that the phosphorylation was sensitive to pertussis toxin, suggesting the role of G_i protein (Gerhardt et al., 1999; Soeder et al., 1999).

In the present study, we examined the effect of toxins which ADP-ribosylate G_s and G_i proteins on H89-sensitive ERK1/2 phosphorylations by β_3-adrenoceptor agonists in 3T3-L1 adipocytes, and demonstrate that the phosphorylations occurred via a pertussis toxin-insensitive but cholera toxin-sensitive pathway. These results suggest that phosphorylations of ERK1/2 by the three β_3-adrenoceptor agonists are induced mainly through the G_s protein-dependent pathway, at least in 3T3-L1 adipocytes.

2. Materials and methods

2.1. Materials

3T3-L1 fibroblast cells (Green and Kehinde, 1976), JCRB 9014 were obtained from The Health Science Research Resources Bank of Japan Health Sciences Foundation. The β_3-adrenoceptor agonist, [(7S)7-{(2R)2-(3-chlorophenyl)-2-hydroxyethyl-amino}-5,6,7,8-tetrahydronapht-2-yl]ethyl oxyacetate, hydrochloride (SR58611) was a kind gift from Dr. Luciano Manara (Research Centre Sanofi Midy) and Dr. Martine Combes (SANOFI RECHERCHE). The following materials were purchased from the sources indicated: Dulbecco's modified Eagle's medium (DMEM) from Nissui; fetal bovine serum from JRH Biosciences; penicillin and streptomycin from Gibco BRL; (±)-(R^*,R^*)-(4-[2-([2-(3-chlorophenyl)-2-hydroxyethyl]amino)propyl]phenoxy)acetic acid (BRL37344) and 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one (CGP12177) from Research Biochemical International; cholera toxin (*Vibrio cholerae*) from List Biological Laboratories; pertussis toxin (*Bordetella pertussis*) from Funakoshi. Other reagents used were of the highest grade commercially available.

2.2. Cell culture and differentiation

3T3-L1 fibroblast cells were maintained in high-glucose DMEM supplemented with 10% fetal bovine serum at 37°C and treated with 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone and 10 mg/ml insulin to initiate adipogenesis as described previously (Mizuno et al., 1999). After 8 days of cultivation, the adipocytes were washed and cultured in the absence of fetal bovine serum for 1 h and then treated with various reagents. In some cases, the adipocytes were pretreated and serum starved in the presence of either pertussis toxin or cholera toxin prior to the experiments. Detailed conditions were described in each result.

2.3. Determination of ERK1 / 2 phosphorylation

Whole cell extract of 3T3-L1 adipocytes were prepared as described previously (Mizuno et al., 1999). Briefly, the adipocytes were washed with ice-cold phosphate-buffered saline containing 1 mM sodium vanadate and 1 mM phenylmethylsulfonyl fluoride, and then gently scraped into cell lysis buffer consisting of 20 mM Tris–HCl (pH 7.5), 10 mM EDTA-2Na, 60 mM β-glycerophosphate, 10 mM $MgCl_2$, 1% Triton X-100, 1 mM dithiothreitol, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin and 2 μg/ml aprotinin. After sonication (1 s × 5 times, on ice) and centrifugation (12,000 × *g*, 20 min, 4°C), the obtained infranatants were used as cell extracts and the protein contents were determined using BCA Protein Assay Reagent Kit (Pierce). The extracts containing 10-μg crude proteins were subjected to sodium

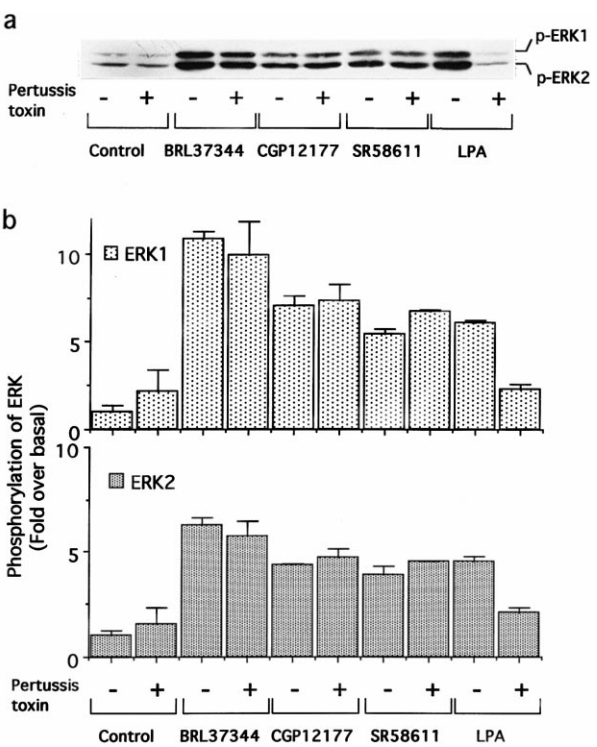

Fig. 1. Effect of pertussis toxin on ERK1/2 phosphorylation induced by β_3-adrenoceptor agonists in 3T3-L1 adipocytes. The 3T3-L1 adipocytes (35 mm dishes, 7 days) were incubated with (+) or without (−) 100 ng/ml pertussis toxin for 24 h at 37°C. After the pretreatment, the adipocytes were serum starved and stimulated by 1 μM BRL37344, 1 μM CGP12177 or 1 μM SR58611 for 30 min, or 10 μg/ml lisophosphatidic acid for 3 min. A typical result of immunoblotting is shown (a). p-ERK1 and p-ERK2 in (a) indicate phosphorylated ERK1 and ERK2, respectively. The increased extents of ERK1/2 phosphorylations (b) were obtained by densimetric quantification of the immunoblots ($n = 3$, values represent means ± S.D.).

dodecyl sulfate–polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinilidene difluoride membrane (IPVH000 10; MILLIPORE), and immunoblotted with anti-phospho specific ERK antibody (#9101, New England Biolabs) as a primary antibody and horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody (Calbiochem-Novabiochem) as a secondary antibody. Bound antibodies were detected by enhanced chemiluminescence (ECL western blotting detection reagents, Amersham) and the intensity of each band was quantitatively analyzed using NIH image ver. 1.61 (National Institute of Health, USA).

3. Results

3.1. Effect of pertussis toxin on ERK1 / 2 phosphorylation induced by the β_3-adrenoceptor agonists in 3T3-L1 adipocytes

As shown in Fig. 1a and b, phosphorylation of ERK1/2 was induced in 3T3-L1 adipocytes by stimulation with β_3-adrenoceptor agonists BRL37344 (1 μM, 30 min), CGP12177 (1 μM, 30 min), and SR58611 (1 μM, 30 min). Lisophosphatidic acid (10 μg/ml, 3 min) has also been seen to cause ERK1/2 phosphorylation in 3T3-L1 adipocytes (Fig. 1a and b) as well as observed in 3T3 fibroblast (Sasaki et al., 1998). The phosphorylations of ERK1/2 by the three β_3-adrenoceptor agonists were not abolished by treating the adipocytes with 100 ng/ml pertussis toxin for 24 h, whereas the phosphorylation of ERK1/2 was almost abolished in the case of lisophosphatidic acid stimulation (Fig. 1a and b).

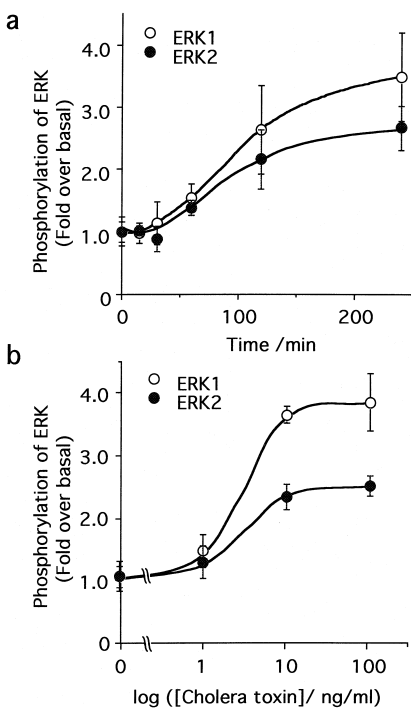

Fig. 2. Time- (a) and dose- (b) dependent ERK1/2 phosphorylation induced by treating 3T3-L1 adipocytes with cholera toxin. The 3T3-L1 adipocytes (35 mm dishes, 8 days) were serum starved and treated with 100 ng/ml cholera toxin for the indicated periods at 37°C (a), or at the indicated concentrations for 4 h (b). The extents of increase were expressed in fold over basal. Values represent means ± S.D. ($n = 3$).

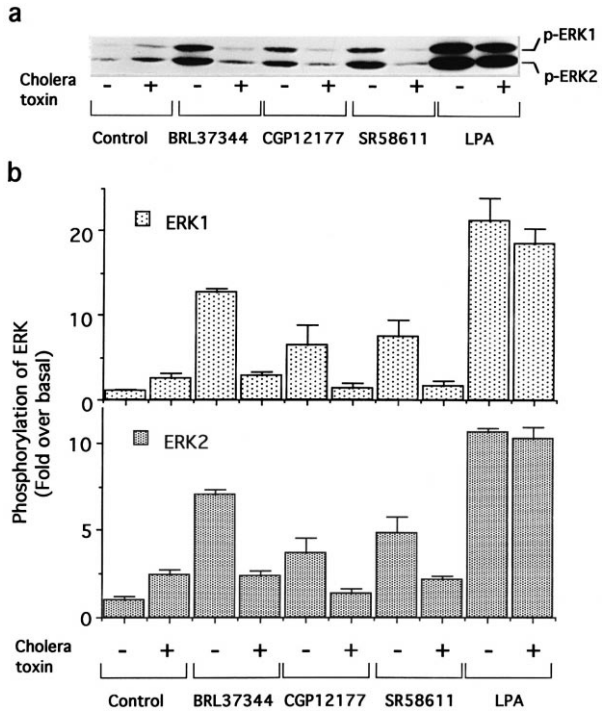

Fig. 3. Change in the extents of ERK1/2 phosphorylation by β_3-adrenoceptor agonists in 3T3-L1 adipocytes after long-term treatment of the adipocyte with cholera toxin. The adipocytes were treated without (−) or with (+) 100 ng/ml cholera toxin for 24 h, serum starved and then stimulated by the β_3-adrenoceptor agonists or lisophosphatidic acid as described in the legend of Fig. 1. A typical result of immunoblotting is shown (a). p-ERK1 and p-ERK2 in (a) indicate phosphorylated ERK1 and ERK2, respectively. The extents of increase of ERK1/2 phosphorylations (b) were obtained by densimetric quantification of the immunoblots ($n = 3$, values represent means ± S.D.).

3.2. Effect of cholera toxin on ERK1 / 2 phosphorylation in 3T3-L1 adipocytes

As shown in Fig. 2a, treatment of the adipocytes with cholera toxin (100 ng/ml) increased the extent of ERK1/2 phosphorylation in a time-dependent manner. The increase became detectable after 1 h of the treatment and was sustained up to, at least, 4 h after the treatment. The time dependency was very similar to glycerol release seen from adipocytes induced by cholera toxin (Vaughan et al., 1970) which is well-known to be mediated by G_s and cAMP-dependent protein kinase. The phosphorylation occurred also in a dose-dependent manner and reached its maximal at 10 ng/ml (Fig. 2b).

3.3. Effect of cholera toxin on ERK1/2 phosphorylation induced by the β_3-adrenoceptor agonists in 3T3-L1 adipocytes

After the treatment of 3T3-L1 adipocytes with 100 ng/ml cholera toxin for 24 h, the ERK1/2 phosphorylations by the three β_3-adrenoceptor agonists were almost completely abolished (Fig. 3a and b). In contrast, lisophosphatidic acid could still effectively increase ERK1/2 phosphorylation to similar extent to that obtained without cholera toxin even after the long-term treatment of the adipocytes with cholera toxin (Fig. 3a and b).

4. Discussion

In the present study, we investigated the roles of G_s and G_i proteins in ERK1/2 phosphorylations by the three β_3-adrenoceptor agonists BRL37344, CGP12177, and SR58611 in 3T3-L1 adipocytes. Treatment of the adipocytes with pertussis toxin (100 ng/ml, 24 h) failed to abolish ERK1/2 phosphorylations by the β_3-adrenoceptor agonists. On the other hand, long treatment of the adipocytes with cholera toxin (100 ng/ml, 24 h) almost completely abolished the ERK1/2 phosphorylations in 3T3-L1 adipocytes. These results implicate that the phosphorylations of ERK1/2 by the β_3-adrenoceptor agonists were mainly mediated by not pertussis toxin-sensitive G_i protein but by cholera toxin-sensitive G_s protein and the downstream pathway(s).

β_3-Adrenoceptors couple to G_s (Guan et al., 1995). Stimulation of the receptors increases intracellular cAMP level (Emorine et al., 1989; Nahmias et al., 1991) and cAMP-dependent protein kinase activity (Langin et al., 1992), and the activated cascade results in physiological events such as lipolysis and inhibition of glucose uptake induced by insulin (Klein et al., 1999; for review, see Strosberg, 1997). β_3-Adrenoceptors are also reported to couple with not only G_s but also with G_i protein functionally in such intact cells as rat primary white adipocytes (Chaudhry et al., 1994) and 3T3-F442A adipocytes (Soeder et al., 1999).

Recently, we and other groups have reported that stimulation of β_3-adrenoceptor caused ERK1/2 phosphorylation (Gerhardt et al., 1999; Mizuno et al., 1999; Soeder et al., 1999). As shown in our previous report, the stimulation of β_3-adrenoceptor increased ERK1/2 phosphorylation in a H89-sensitive manner in 3T3-L1 adipocytes, implying the participation of activated G_s protein and its downstream elements including cAMP-dependent protein kinase in the phosphorylation (Mizuno et al., 1999). In contrast, the studies with CHO cells (Gerhardt et al., 1999) and HEK293 cells (Soeder et al., 1999) expressing human β_3-adrenoceptors as well as a study with intact 3T3-F442A adipocytes (Soeder et al., 1999) showed that stimulation of the β_3-adrenoceptor induced ERK1/2 phosphorylation in a pertussis toxin-sensitive G_i-dependent manner rather than in a G_s-dependent manner, despite the evident increase of cAMP in the cells (Emorine et al., 1989; Nahmias et al., 1991; Gerhardt et al., 1999; Soeder et al., 1999).

First of all, we examined the effect of pertussis toxin on ERK1/2 phosphorylation in 3T3-L1 adipocytes evoked by the three β_3-adrenoceptor agonists BRL37344, CGP12177, and SR58611, and found that the phosphorylations were not abolished by pretreating the adipocytes with 100 ng/ml pertussis toxin for 24 h (see Fig. 1a and b). Lisophosphatidic acid has been reported to mediate ERK1/2 phosphorylation in a G_i protein-dependent manner (Van Corven et al., 1993), and ERK1/2 phosphorylation also occurs in 3T3-L1 adipocytes by means of stimulation with lisophosphatidic acid. In addition, the phosphorylation of ERK1/2 caused by stimulation with lisophosphatidic acid was completely abolished by the long-term treatment of the adipocytes with pertussis toxin, suggesting an indispensable role of pertussis toxin-sensitive G_i protein in lisophosphatidic acid-induced ERK1/2 phosphorylation in 3T3-L1 adipocytes. The results regarding lisophosphatidic acid were not only consistent with the previous report (Van Corven et al., 1993), but still provide important information on the signaling pathway in the 3T3-L1 adipocytes, showing that the adipocytes contain functioning pertussis toxin-sensitive G protein, namely, G_i protein, and a signaling pathway leading to ERK1/2 phosphorylation via the G_i protein. These results obtained with pertussis toxin suggest that stimulation by the β_3-adrenoceptor agonists does evoke ERK1/2 phosphorylation in 3T3-L1 adipocytes through a pertussis toxin-insensitive pathway but not a pertussis toxin-sensitive G_i protein-dependent pathway.

Cholera toxin is known to cause ADP-ribosylation of G_s protein at specific amino acid residues and to impair the hydrolyzing activity toward GTP by the modification. The disactivation of GTP-hydrolyzing activity leads to irreversible activation of G_s protein, resulting in activation of downstream molecules such as adenylate cyclase and cAMP-dependent protein kinase (Cassel and Selinger, 1997). To elucidate the role of G_s protein in ERK1/2 phosphorylation in 3T3-L1 adipocytes, the effects of cholera toxin on the phosphorylations were examined. Treatment of the adipocytes with cholera toxin resulted in an increase of ERK1/2 phosphorylation in a time- and dose-dependent manner (Fig. 2a and b). The time course was similar to that of glycerol release by cholera toxin in adipocytes (Vaughan et al., 1970), which was well-known to be dependent on the activation of G_s protein. These results suggest, at least, that activation of G_s protein is able to cause ERK1/2 phosphorylation, though the phosphorylation fold of ERK1/2 by cholera toxin seemed somewhat lower than those induced by the β_3-adrenoceptor agonists.

It has been reported that in various cells, long-term treatment with cholera toxin caused the down-regulation of functional G_s protein (Milligan et al., 1989; Chang and

Bourne, 1989; Carr et al., 1990) and abolished G_s-coupled receptor signaling (Sexl et al., 1997, Seidel et al., 1999). As shown in Fig. 3a and b, the long exposure of 3T3-L1 adipocytes to cholera toxin almost completely abolished the phosphorylation of ERK1/2 by the three β_3-adrenoceptor agonists in the adipocytes. On the other hand, the effect of lisophosphatidic acid to evoke ERK1/2 phosphorylation was not altered by the long-term treatment (Fig. 3a and b), suggesting that the ability of cholera toxin to block ERK1/2 phosphorylation by the β_3-adrenoceptor agonists is not due to non-specific toxicity of cholera toxin but to the selective down-regulation of G_s protein.

Taken together, the results obtained in the present studies and those of our previous report showing that the ERK1/2 phosphorylations by the β_3-adrenoceptor agonists were H89-sensitive (Mizuno et al., 1999), we propose that the β_3-adrenoceptor agonists BRL37344, CGP12177, and SR58611 do induce ERK1/2 phosphorylation via a pathway involving G_s protein, adenylate cyclase, and cAMP-dependent protein kinase but not via a G_i protein-dependent pathway, at least in 3T3-L1 adipocytes. The differences between the present report and the previous reports might be due to qualitative and/or quantitative variations of particular molecules that might participate in G_s protein-dependent ERK1/2 phosphorylation, such as cAMP-dependent protein kinase, Rap-1, B-Raf (Vossler et al., 1997; Wan and Huang, 1998) and cAMP-regulated guanine nucleotide exchange factors (Kawasaki et al., 1998), although the accurate mechanisms of the phosphorylations remains to be clarified. This interesting phenomenon that the β_3-adrenoceptors may induce ERK1/2 phosphorylation in either G_s- or G_i-dependent manner under certain circumstances might indicate the indispensable roles of ERK1/2 phosphorylation in diverse β_3-adrenoceptor-mediated responses.

Acknowledgements

We thank Dr. Luciano Manara of Research Centre Sanofi Midy and Dr. Martine Combes of SANOFI RECHERCHE for the β_3-adrenoceptor agonist SR58611. This work was supported in part by a grant to Y.W. from the Smoking Research Foundation.

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