

Stimulation of β_3 -adrenoceptors causes phosphorylation of p38 mitogen-activated protein kinase *via* a stimulatory G protein-dependent pathway in 3T3-L1 adipocytes

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1 This study deals with phosphorylation and activation of p38 mitogen-activated protein kinase (MAPK) *via* β_3 -adrenoceptor (AR) and the signal transduction pathway in 3T3-L1 adipocytes.

2 β_3 -AR agonist BRL37344A (10 nM) caused phosphorylation and activation of p38 MAPK in 3T3-L1 adipocytes but not in fibroblasts. BRL37344A and also the other β_3 -AR agonists, CGP12177A and SR58611A, caused p38 MAPK phosphorylation in dose-dependent manners.

3 The p38 MAPK phosphorylations by BRL37344A (10 nM), CGP12177A (100 nM), and SR58611A (10 nM) were not antagonized by β_1 - and β_2 -ARs antagonist 1-propranolol (100 nM) but blocked by β_3 -AR antagonist SR59230A (10 μ M), suggesting the phosphorylation was caused *via* β_3 -AR.

4 The phosphorylations of p38 MAPK were completely abolished by treatment with cholera toxin (CTX) but not pertussis toxin (100 ng ml⁻¹, 24 h). Activation of Gs by CTX (100 ng ml⁻¹) and adenylyl cyclase by forskolin mimicked p38 MAPK phosphorylation.

5 p38 MAPK phosphorylation by BRL37344A was reduced to almost 50% by cyclic AMP-dependent protein kinase (PKA) inhibitors such as H89 (10 μ M) and PKI (10 μ M). A src-family tyrosine kinases inhibitor PP2 (1 μ M) also halved the p38 MAPK phosphorylation. Combined use of H89 (10 μ M) and PP2 (10 μ M) did not bring about further inhibition.

6 These results suggest that β_3 -AR caused phosphorylation of p38 MAPK *via* Gs protein and partly through a pathway involving PKA and src-family kinase(s), although the contribution of the unidentified pathway remains to be clarified.

British Journal of Pharmacology (2002) **135**, 951–960

Keywords: β_3 -adrenoceptors; p38 MAPK; 3T3-L1 adipocytes; BRL37344A; CGP12177A; SR58611A; SR59230A

Abbreviations: β -AR, β -adrenoceptor; BSA, bovine serum albumin; BRL37344A, *dl*-4-[2'-(2-hydroxy-2-(3-chlorophenyl)ethylamino)propyl phenoxyacetic acid sodium salt sesquihydrate; CGP12177A, *dl*-4-3[(1,1-dimethylethyl)amino]-(2-hydroxypropoxy)1,3-dihydro-2H-benzimidazol-2-one hydrochloride; CTX, cholera toxin; DMEM, Dulbecco's modified Eagle medium; ERK, extra cellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PTX, pertussis toxin; SR58611A, [(7S)-{(2R)-2-(3-chlorophenyl)-2-hydroxyethyl-amino}-5,6,7,8-tetrahydronaph-2-yl]ethyl oxyacetate, hydrochloride; SR59230A, SS-enantiomer 3-(2-ethylphenoxy)-1-[(1S)-1,2,3,4-tetrahydronaph-1-ylaminol]-(2S)-2-propanol oxalate

Introduction

β_3 -Adrenoceptors (ARs) are expressed predominantly, though not exclusively, in white and brown adipocytes (Emorine *et al.*, 1989; Granneman *et al.*, 1991; Muzzin *et al.*, 1991; Nahmias *et al.*, 1991). Treatment of animals with β_3 -AR selective agonists causes a number of diverse metabolic effects including lipolysis (Van Liefde *et al.*, 1992), increase in energy expenditure (Weyer *et al.*, 1998), reduction of food intake (Mantzoros *et al.*, 1996), and dramatic increase of insulin level in blood (Susulic *et al.*, 1995). These effects have been shown to disappear in β_3 -AR knock-out mice (Susulic *et al.*, 1995). Re-expression of the receptors in both white and brown adipocytes of the knock-out mice recovered these effects, whereas re-expression of the receptors only in brown adipocytes did not

(Grujic *et al.*, 1997). This interesting phenomenon strongly suggests the significance of β_3 -AR-mediated responses in the functioning white adipocytes. In addition, it has been reported that amelioration of type 2 diabetes was caused by treatment with β_3 -AR agonists; thus, β_3 -AR agonists are expected to act as anti-obesity and/or anti-diabetic compounds in humans as well (Weyer *et al.*, 1998; Sum *et al.*, 1999; Kato *et al.*, 2001). Despite its increasingly apparent significance, however, our understanding of the signalling pathway of β_3 -AR in adipocytes is insufficient, suggesting the need for further investigation of the signalling of β_3 -AR in order to realize an efficient treatment for obesity and/or insulin resistance (Klein *et al.*, 1999).

Mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase 1 and 2 (ERK1/2) and p38 MAPK play key roles in many physiological events, such

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as proliferation (Pages *et al.*, 1993; Kanda *et al.*, 2001), differentiation (Pang *et al.*, 1995; Engelman *et al.*, 1998), cell survival (Lindquist & Rehnmark, 1998, Kuroki *et al.*, 2000) and gene expression (Vanhoute *et al.*, 1998). Recently, it was found that stimulation of β_3 -AR caused phosphorylation and activation of ERK1/2 in brown adipocytes (Shimizu *et al.*, 1997; Lindquist & Rehnmark, 1998; Lindquist *et al.*, 2000), 3T3-L1 adipocytes (Mizuno *et al.*, 1999; 2000), 3T3-F442A (Soeder *et al.*, 1999), and exogenous β_3 -AR expression system with CHO/K1 cells (Gerhardt *et al.*, 1999) or HEK293 cells (Soeder *et al.*, 1999), although the physiological functions of this novel signalling pathway have been clarified only limitedly in brown adipocytes (Lindquist & Rehnmark, 1998). In addition, the pathway leading to ERK1/2 activation and phosphorylation, which includes coupling of G proteins to β_3 -AR, and the modulation mechanisms are controversial (Gerhardt *et al.*, 1999; Lindquist *et al.*, 2000; Mizuno *et al.*, 2000; Soeder *et al.*, 1999; Cao *et al.*, 2000) and remain insufficiently elucidated. In the complexity of the β_3 -AR signalling pathways, very little is known about the activation of p38 MAPK via β_3 -AR. The β -AR agonist isoproterenol has been shown to cause activation of p38 MAPK in freshly isolated white adipocytes of rat (Moule & Denton, 1998), whereas a study with CGP12177A, a β_3 -AR agonist, failed to obtain clear phosphorylation of p38 MAPK in CHO/K1 cells which expressed exogenous β_3 -AR (Gerhardt *et al.*, 1999). Very recently, it was confirmed by the study with C3H10T1/2 adipocytes and brown adipocytes that a β_3 -AR agonist CL316,243 caused p38 MAPK phosphorylation and that the phosphorylation was completely inhibited by treatment with a PKA inhibitor H89 (Cao *et al.*, 2001).

In this study, we investigated whether selective β_3 -AR agonists had the potential to induce p38 MAPK phosphorylation using 3T3-L1 fibroblasts and 3T3-L1 adipocytes. We also examined the downstream pathway of β_3 -AR that leads to phosphorylation and activation of p38 MAPK including participations of G proteins, and found that PKA and src-family tyrosine kinase(s) were likely to participate in phosphorylation of p38 MAPK.

Methods

Materials

The 3T3-L1 fibroblast cells (Green & Kehinde, 1976), JCRB 9014, were obtained from The Health Science Research Resources Bank of the Japan Health Sciences Foundation. The β_3 -AR antagonist SR59230A and agonist SR58611A were the kind gifts of 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 medium (DMEM) from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan); foetal bovine serum (FBS) from JRH Biosciences (Lenexa, KS, U.S.A.); penicillin and streptomycin from Life technologies (Grand Island, NY, U.S.A.); BRL37344A and CGP12177A from Research Biochemical International (Natick, MA, U.S.A.); cholera toxin (CTX) of *Vibrio cholerae* from List Biological Laboratories, Inc. (Campbell, CA, U.S.A.); pertussis toxin (PTX) of *Bordetella pertussis* from Seikagaku Corporation (Tokyo, Japan). H89 (N-[2-(p-

bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride), PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) and cell-permeable cyclic AMP-dependent protein kinase inhibitor peptide (PKI-(14–22)-amide) were from Calbiochem-Novabiochem Corporation (La Jolla, CA, U.S.A.). Other reagents used were of the highest grade commercially available.

Cell culture and differentiation

3T3-L1 fibroblast cells were maintained in high-glucose (25 mM) DMEM supplemented with 10% FBS at 37°C (95% air/5% CO₂) and treated with 0.5 mM 3-isobutyl-1-methylxanthine, 1 mM dexamethasone and 10 mg ml⁻¹ insulin to initiate adipogenesis as described previously (Mizuno *et al.*, 1999). After appropriate cultivation, the adipocytes were washed and cultured in the absence of FBS for 4 h and then treated with various reagents. To confirm effect of SR59230A, 3T3-L1 adipocytes were cultured in glass dishes to avoid binding of SR59230A to the walls, as it does to plastic culture dishes (Nisoli *et al.*, 1996). In some cases, the adipocytes were pretreated and serum-starved in the presence of either PTX or CTX prior to the experiments. The conditions are described in detail in the results for each trial.

Immunoblot analysis

Whole-cell extracts of 3T3-L1 cells were prepared as described previously (Mizuno *et al.*, 1999). Briefly, the cells were washed with ice-cold phosphate-buffered saline containing 1 mM sodium vanadate and 1 mM phenylmethylsulfonyl fluoride (PMSF), and then gently scraped into cell lysis buffer consisting of in mM: Tris-HCl (pH 7.5) 20, EDTA-2Na 10, β -glycerophosphate 60, MgCl₂ 10, Triton X-100 1%, dithiothreitol 1, sodium vanadate 1, PMSF 1, leupeptin 2 μ g ml⁻¹ and aprotinin 2 μ g ml⁻¹. After sonication (1 s \times 5 times, on ice) and centrifugation (12,000 \times g, 20 min, 4°C), the obtained supernatants of fibroblasts or infranatants of adipocytes were used as cell extracts.

To prepare membrane fractions, the washed cells were scraped and homogenized in ice-cold homogenizing buffer consisting of in mM Tris-HCl (pH 7.5) 25, EDTA-2Na 1, 5 μ g ml⁻¹ leupeptin, 1 μ g ml⁻¹ aprotinin, and PMSF 1. The homogenates were centrifuged (500 \times g, 10 min, 4°C) and the recovered supernatant was centrifuged again (40,000 \times g, 10 min, 4°C). The resultant pellets were suspended and used as membrane fractions.

The protein contents of these fractions were determined using a BCA Protein Assay Reagent Kit from Pierce (Rockford, IL, U.S.A.).

The cell extracts or membrane fractions containing 10 μ g of crude proteins were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene difluoride membrane (IPVH00010; Millipore, Bedford, MA, U.S.A.), and immunoblotted with anti-phospho p38 MAPK (Thr180/Tyr182) specific antibody (number 9210; New England Biolabs, Beverly, MA, U.S.A.), anti-p38 MAPK antibody (number sc-383; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), or anti- β_3 -AR antibody (sc-1473; Santa Cruz Biotechnology), and then with horseradish peroxidase-conjugated anti-rabbit

IgG antibody (Calbiochem-Novabiochem, La Jolla, CA, U.S.A.) or anti-goat IgG (ICN Biomedicals, Inc. Aurora, OH, U.S.A.) as a secondary antibody. Bound antibodies were detected by enhanced chemiluminescence using ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) and photographed using Fuji X-ray film (Fuji Film Co., Tokyo, Japan). The intensity of each band was quantitatively analysed using NIH image software ver. 1.61 (National Institute of Health).

Activation of p38 MAPK was assayed with a p38 MAP Kinase Assay Kit (number 9820; New England Biolabs).

Statistical analysis

Each experiment was performed independently at least three times in triplicate. The data within groups were compared using an analysis of variance (one way-ANOVA) with Dunnett's or Tukey's *post-hoc* correction for multiple comparisons. Detailed condition was shown in each result.

Results

Stimulation with β_3 -AR agonists induced p38 MAPK phosphorylation in 3T3-L1 adipocytes, but not in fibroblasts

Stimulation with the β_3 -AR agonist BRL37344A did not cause phosphorylation of p38 MAPK in either 3T3-L1 fibroblasts or the cells, when given immediately after the initiation of adipogenesis (Figure 1a,b). On the other hand, when administrated 5 days or more after the initiation of adipogenesis, the stimulation induced clear and statistically significant increases in the phosphorylation levels of threonine (180) and tyrosine (182) residues of p38 MAPK (Figure 1a,b). The phosphorylated p38 MAPK showed the ability to phosphorylate ATF-2 (Figure 1b).

The increase in phosphorylation fold of p38 MAPK by the stimulation with BRL37344A occurred in a time-dependent manner. The stimulation increased the phosphorylation level of p38 MAPK, reached a maximal level at 15 min, and was sustained for at least 120 min after the stimulation (Figure 2a). The protein contents of p38 MAPK were not changed over this time period (see Figure 1b). As summarized in Figure 2b, BRL37344A and the other β_3 -AR agonists, CGP12177A and SR58611A, increased the degree of p38 MAPK phosphorylation in a dose-dependent manner.

Effect of β -AR antagonists on the phosphorylation of p38 MAPK in 3T3-L1 adipocytes

As shown in Figure 3a, the β_1 - and β_2 -ARs specific antagonist *l*-propranolol (100 nM) did not affect the degree of p38 MAPK phosphorylation caused by BRL37344A (10 nM), CGP12177A (100 nM) or SR58611A (10 nM). On the other hand, the β_3 -AR specific antagonist SR59230A (Manara *et al.*, 1996) decreased the phosphorylation of p38 MAPK caused by BRL37344A in a dose-dependent manner and almost completely blocked it at a concentration of 10 μ M (Figure 3b). In addition, the phosphorylations of p38 MAPK by CGP12177A and SR58611A were also decreased to the unstimulated level by the β_3 -AR antagonist at this concen-

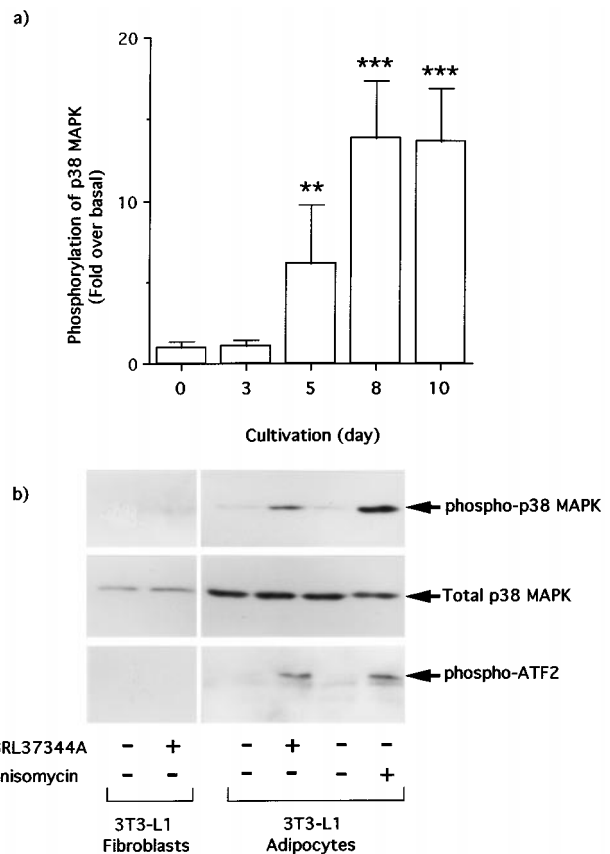


Figure 1 Cultivation-dependent occurrence of p38 MAPK phosphorylation and activation by the stimulation with BRL37344A in 3T3-L1 cells. The 3T3-L1 fibroblast cells were grown and treated with differentiation reagents for initiation of adipogenesis. After appropriate cultivation, the cells were serum-starved and stimulated with 10 nM BRL37344A for 30 min at 37°C. Open bars represent the degree of p38 MAPK phosphorylation at each period, expressed as the fold increase in phosphorylation level over respective basal level (a). Values represent the means \pm s.d. (four independent experiments). The values are significantly different from that obtained at day 0 by one-way ANOVA and Dunnett's multiple comparison (**: $P < 0.01$, ***: $P < 0.001$). The activation of p38 MAPK was confirmed at 8-day cultivation after the initiation by immunoprecipitation kinase assay with ATF-2 as described in the Methods, and representative results are shown (b). A positive control was established using 10 μ M anisomycin under the same condition.

tration (Figure 3c), although a slight increase in p38 MAPK phosphorylation was seen even in the absence of agonist(s) (Figure 3b,c).

Effects of bacterial toxins on the phosphorylations of p38 MAPK by β_3 -AR agonists

Long-term treatment with CTX dramatically decreased immuno-reactive Gs protein contents in 3T3-L1 adipocytes (Figure 4a,b) without effects on the contents of p38 MAPK and β_3 -AR on membrane of the adipocytes (Figure 4a). At the same time, the increases in phosphorylations of p38 MAPK by BRL37344A (10 nM), CGP12177A (100 nM), and SR58611A (10 nM) were completely abolished by the treatment (Figure 4c), whereas lysophosphatidic acid (LPA) has been shown to remain effective at increasing ERK1/2

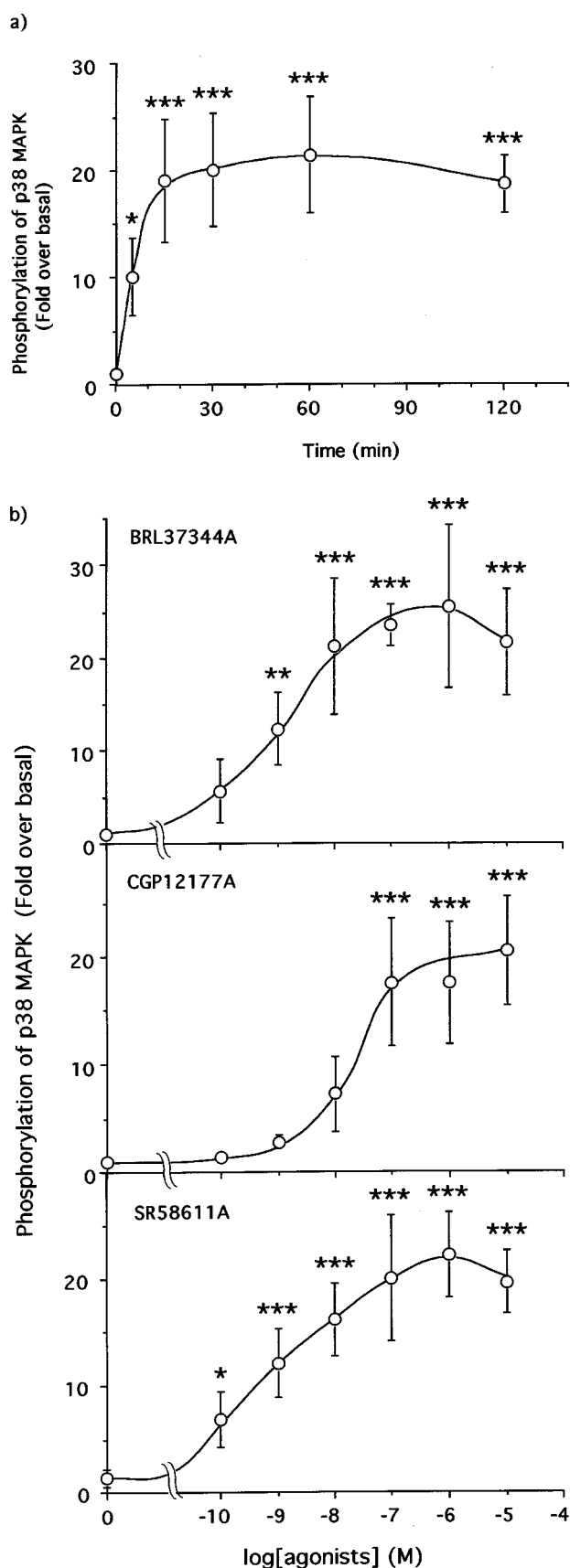


Figure 2 Time- (a) and dose-dependency (b) of p38 MAPK phosphorylation by BRL37344A in 3T3-L1 adipocytes. 3T3-L1 adipocytes (35 mm dish, 8-day culture) were treated for the indicated

phosphorylations after the same treatment (Mizuno *et al.*, 2000). On the other hand, as shown in Figure 4d, treatment with PTX failed to block the p38 MAPK phosphorylations caused by BRL37344A (10 nM), CGP12177A (100 nM), and SR58611A (10 nM), whereas ERK1/2 phosphorylations by LPA (10 μ M) were completely abolished (Mizuno *et al.*, 2000).

Phosphorylation of p38 MAPK by activation of the Gs-dependent pathway

Treatment of 3T3-L1 adipocytes for several hours with CTX, which is known as an activator of Gs protein (Cassel & Selinger, 1997), increased p38 MAPK phosphorylation in a time- and dose-dependent manner (Figure 5a,b).

Forskolin, by which adenylyl cyclase (AC) is directly activated (Seamon & Daly, 1981), also caused p38 MAPK phosphorylation in 3T3-L1 adipocytes (Figure 5c,d).

Phosphorylation of p38 MAPK by the β_3 -AR agonists is mediated via a pathway involving PKA and src-family tyrosine kinase(s)

As shown in Figure 6a, treatment of the adipocytes with H89, the highly selective inhibitor for cyclic AMP-dependent protein kinase (PKA), decreased the phosphorylation of p38 MAPK in a dose-dependent manner, achieving a maximal reduction of approximately 50% at a concentration of 10 μ M. In addition, another PKA inhibitor, PKI-(14–22)-amide also decreased the phosphorylation of p38 MAPK in a dose-dependent manner and almost halved the p38 MAPK phosphorylation at 10 μ M (Figure 6b). Treatment with a src-family tyrosine kinases inhibitor, PP2, also decreased the phosphorylation of p38 MAPK by BRL37344A in a dose-dependent manner, and also reached a maximal reduction of about 50% (Figure 6c). Combined use of 10 μ M H89 and 10 μ M PP2 did not enhance the decrease in phosphorylation of p38 MAPK by 10 nM of BRL37344A (Figure 6d).

Discussion

We here showed that stimulation of β_3 -AR caused phosphorylation of p38 MAPK in 3T3-L1 adipocytes *via* Gs but not Gi protein, and that the downstream pathway of this phosphorylation may have involved AC, PKA and src-family tyrosine kinase(s).

As shown in Figure 1a,b, the β_3 -AR agonist BRL37344A was effective at inducing p38 MAPK phosphorylation and activation in 3T3-L1 adipocytes, whereas it was not effective in 3T3-L1 fibroblasts that did not express β_3 -AR (Mizuno *et al.*, 1999). The activation of p38 MAPK by the phosphoryla-

periods with 10 nM BRL37344A (a) or for 30 min at the indicated concentrations with either BRL37344A, CGP12177, or SR58611A (b) at 37°C. The increases of phosphorylation were measured as described in Figure 1. Open circles represent the results obtained at each period or with each agonist (means \pm s.d. of three independent experiments). The data were compared with the values obtained without agonists as controls by one-way ANOVA and Dunnett's multiple comparison (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

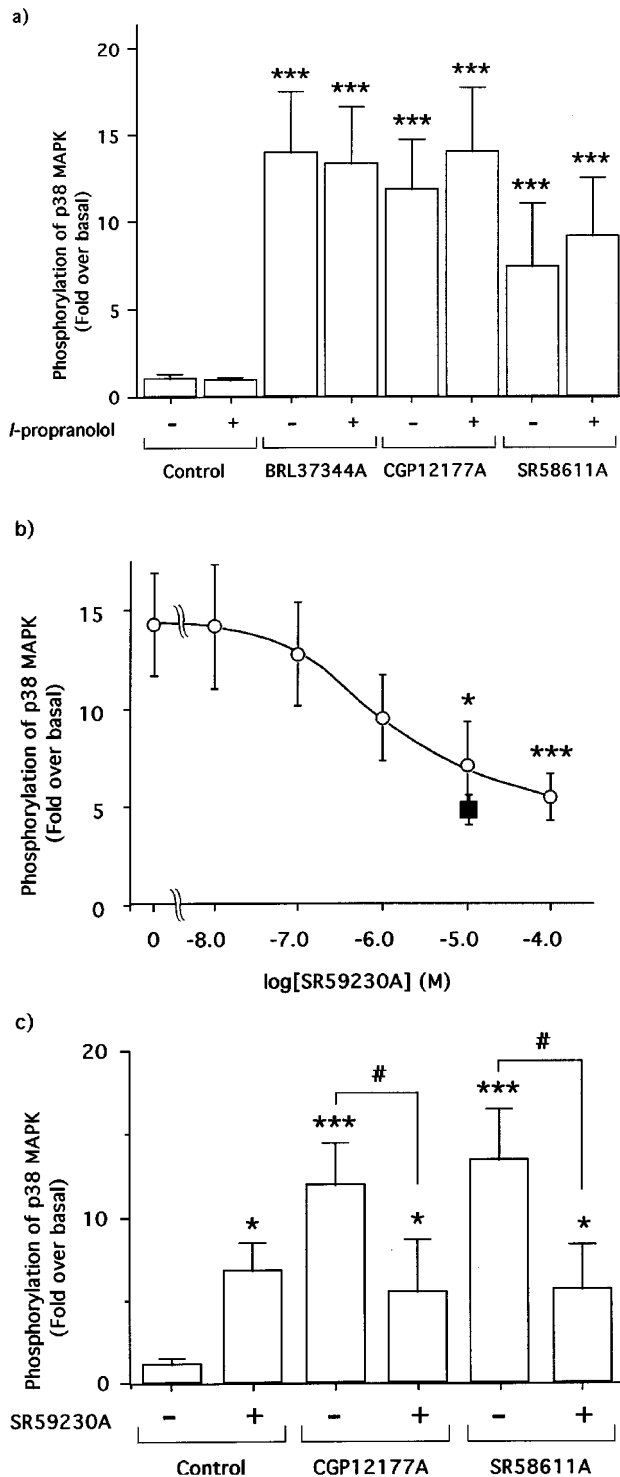


Figure 3 Effects of β -AR antagonists on p38 MAPK phosphorylations by the β_3 -AR agonists. 3T3-L1 adipocytes (35 mm dish, 8-day cultivation) were treated with 10 nM BRL37344A, 100 nM CGP12177A and 10 nM SR58611A in the absence or presence of 100 nM *l*-propranolol (a). The adipocytes were cultured in glass dishes and stimulated by 10 nM BRL37344A for 30 min in the presence of SR59230A at the indicated concentrations (b) or by 100 nM CGP12177A and 10 nM SR58611A for 30 min in the presence of 10 μ M SR59230A (c). The increases of p38 MAPK phosphorylations were measured and expressed as described in Figure 1 (means \pm s.d. of three independent experiments). Open bars in (a,c) represent the results obtained with/without each antagonist. The open circles and closed square in (b) represent the results obtained with 10 nM BRL37344A and SR59230A at the indicated

tion was confirmed by immunoprecipitation kinase assay (Figure 1b). The result was consistent with the previous report that p38 MAPK was activated by the phosphorylation of these residues (Derijard *et al.*, 1995).

The kinetics of p38 MAPK phosphorylation by BRL37344A in 3T3-L1 adipocytes and that of activation by isoproterenol in rat white adipocytes (Moule & Denton, 1998) were well consistent with each other. Both results showed sustained activation with a peak at 15–30 min after stimulation.

As shown in Figure 3a, the β_1 - and β_2 -ARs-specific antagonist *l*-propranolol did not affect the degree of p38 MAPK phosphorylation caused by BRL37344A (10 nM), CGP12177A (100 nM) or SR58611A (10 nM) despite the fact that this concentration was previously reported to be sufficient to completely block β_1 - and β_2 -ARs (Arch *et al.*, 1984). On the other hand, the β_3 -AR-specific antagonist SR59230A decreased the BRL37344A-induced phosphorylation of p38 MAPK in a dose-dependent manner and nearly reached the unstimulated level at a concentration of 10 μ M (Figure 3b). SR59230A also completely blocked the p38 MAPK phosphorylations by CGP12177A and SR58611A (Figure 3c), whereas SR59230A only slightly blocked the stimulation of β_1 - and β_2 -ARs by specific agonists at this concentration (Nisoli *et al.*, 1996). These results strongly suggest that the phosphorylations of p38 MAPK in 3T3-L1 adipocytes by BRL37344A, CGP12177A and SR58611A were caused *via* β_3 -AR, rather than by β_1 - or β_2 -ARs.

It has previously been shown that β_3 -ARs are coupled to Gs protein (Guan *et al.*, 1995; Soeder *et al.*, 1999). Thus, stimulation of the receptors rapidly activates the Gs protein and Gs-coupled AC. The activated AC generates cyclic AMP (Emorine *et al.*, 1989; Nahmias *et al.*, 1991), leading to activation of cyclic AMP-dependent protein kinase (PKA). The lipolysis, one of the most characteristic responses mediated through β_3 -AR in white adipocytes, is induced *via* activation of hormone-sensitive lipase by PKA (Langin *et al.*, 1992; Anthonen *et al.*, 1998). However, some studies have provided evidence that β_3 -AR receptors also constitutively couple to Gi protein in adipocytes (Chaudhry *et al.*, 1994; Soeder *et al.*, 1999) and the heart (for review see Gauthier *et al.*, 2000). Recently, it has been confirmed that stimulation of β_3 -AR causes phosphorylation and activation of ERK1/2 in brown adipocytes (Lindquist *et al.*, 2000) and 3T3-L1 adipocytes (Mizuno *et al.*, 1999; 2000) through a Gs-protein-dependent pathway. On the other hand, it has been reported that ERK1/2 activation *via* β_3 -AR was caused through pertussis toxin (PTX)-sensitive Gi protein in 3T3-F442A adipocytes (Soeder *et al.*, 1999) as well as CHO/K1 cells (Gerhardt *et al.*, 1999) and HEK293 cells (Soeder *et al.*, 1999) expressing exogenous β_3 -AR. As mentioned above, the complicated signal-transduction pathways of β_3 -AR, including the coupling of β_3 -AR to G protein(s), are insufficiently

concentrations or that with 10 μ M SR59230A alone, respectively. The data in (a) and (c) were compared with the controls (*: P <0.05, **: P <0.001) or each other (#: P <0.001) by one-way ANOVA and Tukey's multiple comparison. The data in (b) were compared with the values obtained with BRL37344A as the control by one-way ANOVA and Dunnett's multiple comparison (*: P <0.05, **: P <0.001).

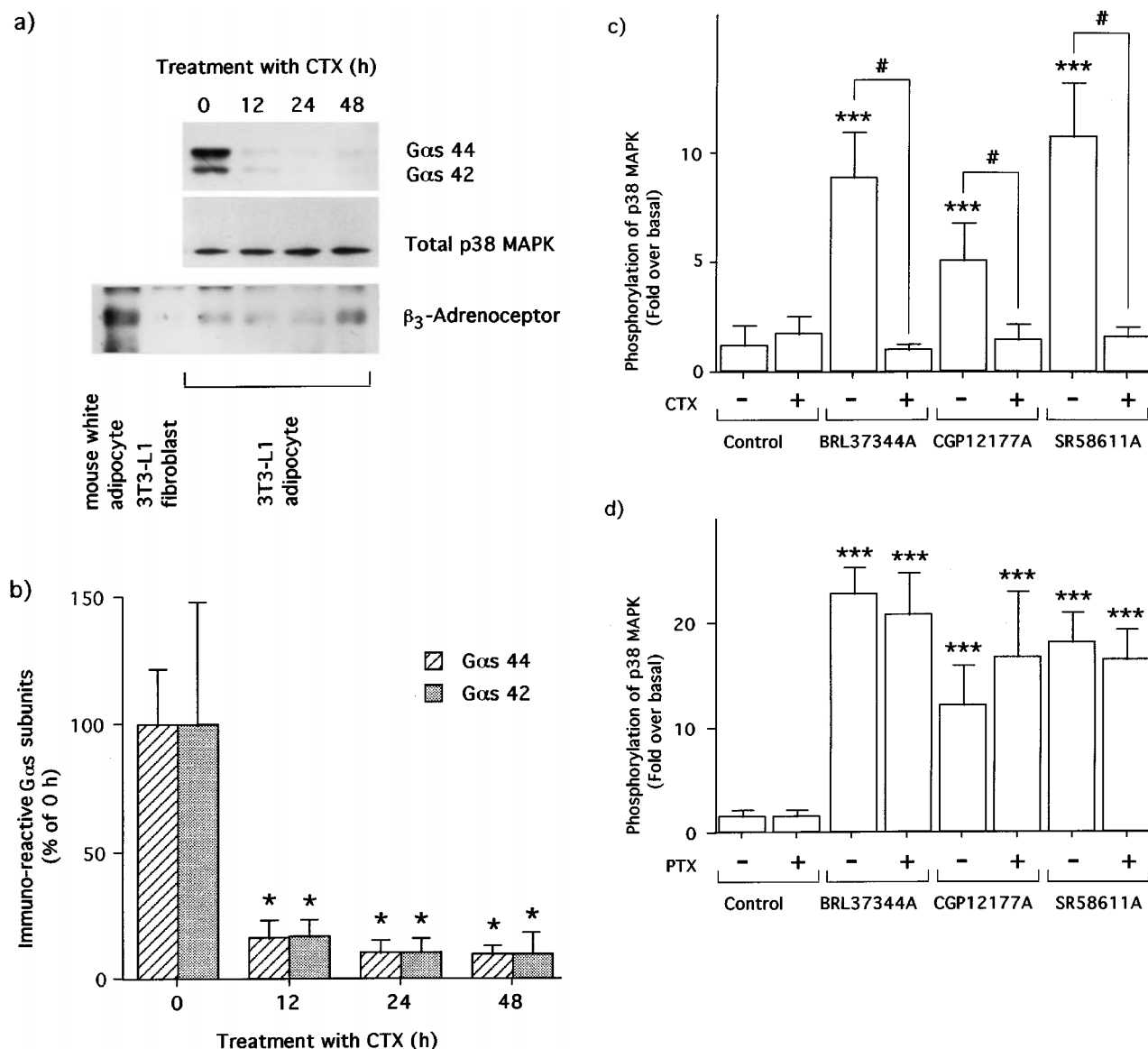


Figure 4 Effects of CTX and PTX on p38 MAPK phosphorylations caused by β_3 -AR agonists in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with 100 ng ml⁻¹ CTX for the indicated periods at 37°C. The contents of immuno-reactive Gs, p38 MAPK and β_3 -AR proteins are shown in upper, middle, and lower panels of (a), respectively. The hatched and shaded bars express the relative contents of two isoforms of Gs proteins in the membrane of 3T3-L1 adipocytes at indicated periods (b). After treatment with 100 ng ml⁻¹ CTX (c) or PTX (d) for 24 h, 3T3-L1 adipocytes were serum-starved and then stimulated by the β_3 -AR agonists as described in the legend for Figure 3. The increases of p38 MAPK phosphorylations were measured as described in Figure 1 and expressed as open bars (means \pm s.d. of three independent experiments). The data in (b) were compared with values obtained at 0 h as the controls by one-way ANOVA and Dunnett's multiple comparison (*: $P < 0.001$). The data in (c) and (d) were compared with the values obtained without agonists (***: $P < 0.001$) or each other (#: $P < 0.001$) by one-way ANOVA with Tukey's multiple comparison.

understood, prompting us to examine what kinds of G protein(s) were involved in the p38 MAPK phosphorylation.

To elucidate the role of Gs and Gi proteins in the p38 MAPK phosphorylations *via* β_3 -AR, we examined the effects of bacterial toxins that selectively impair the individual signalling of each G protein. It has been reported that long-term treatment with CTX caused selective down regulation of Gs proteins and blocked signalling of Gs protein-coupled receptors in various cells (Chang & Bourne, 1989; Milligan *et al.*, 1989; Carr *et al.*, 1990). These phenomena were also found in 3T3-L1 adipocytes. Treatment of the adipocytes

with CTX (100 ng ml⁻¹) for 24 h was sufficient to bring almost complete reduction of Gs proteins (Figure 4a,b). The total content of p38 MAPK in the cells and that of β_3 -AR in the membrane fractions remained almost unchanged even after the treatments (Figure 4a). As shown in Figure 4c, the stimulation of 3T3-L1 adipocytes by BRL37344A (10 nM), CGP12177A (100 nM) and SR58611A (10 nM) did not increase phosphorylation of p38 MAPK after long-term treatment with CTX. These results suggest that Gs protein plays an important role in the phosphorylation of p38 MAPK by the three β_3 -AR specific agonists in 3T3-L1

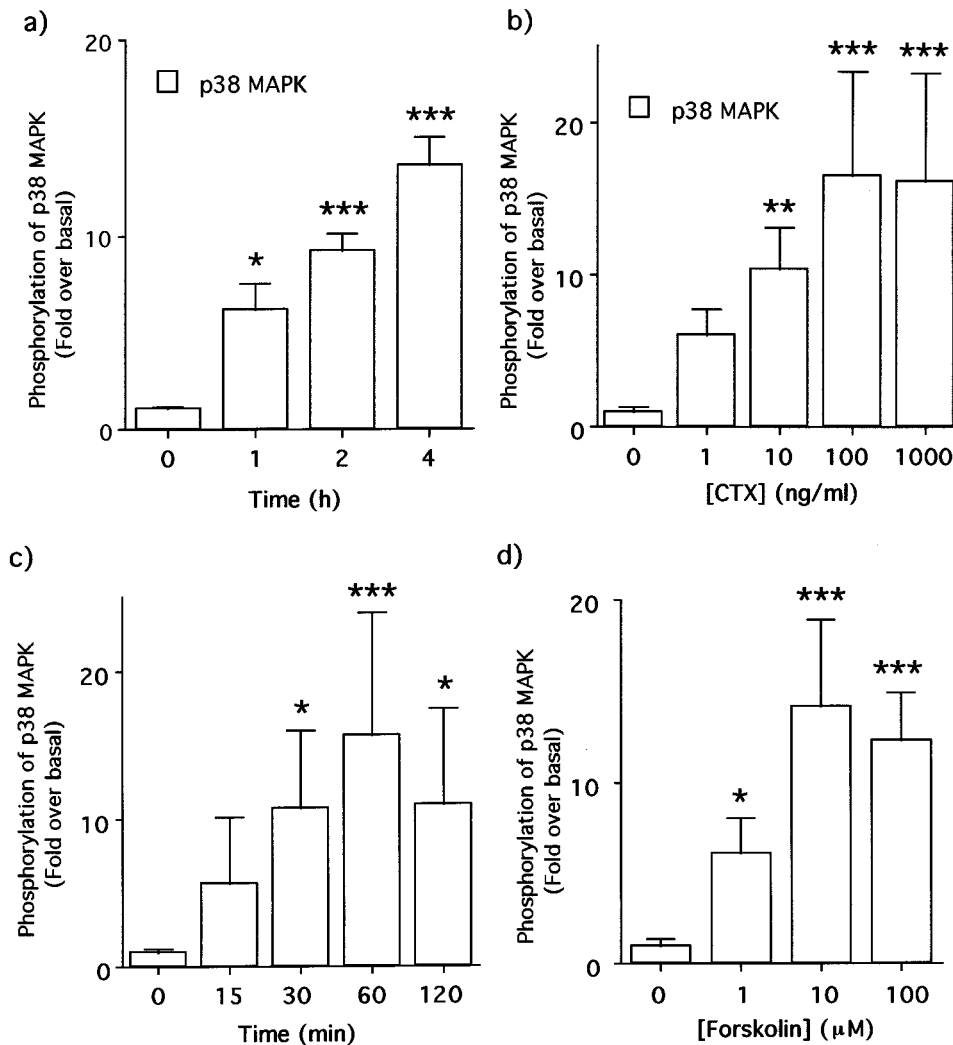


Figure 5 p38 MAPK phosphorylations in 3T3-L1 adipocytes by CTX (a and b) and forskolin (c and d). 3T3-L1 adipocytes were treated with 100 ng ml⁻¹ CTX (a) or 1 μM forskolin (c) for the indicated periods and at the indicated concentrations for 4 h with CTX (b) or for 30 min with forskolin (d). The increases of p38 MAPK phosphorylations were measured as described in Figure 1 and expressed as open bars (means ± s.d. of three independent experiments). The data were compared with the values obtained at 0 min or 0 h without reagents as the control by one-way ANOVA with Dunnett's multiple comparison (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

adipocytes. On the other hand, long-term treatment of the cells with PTX (100 ng ml⁻¹), which is known to diminish Gi protein-dependent signalling (van Corven *et al.*, 1993), did not alter the phosphorylation of p38 MAPK caused by the β_3 -AR agonists (Figure 4d), whereas phosphorylations of ERK1/2 by LPA that occurred through Gi protein was completely abolished by the same treatment even in 3T3-L1 adipocytes (Mizuno *et al.*, 2000). These results suggest that the Gs protein rather than the Gi protein plays an indispensable role in p38 MAPK phosphorylation caused by the stimulation of β_3 -AR in 3T3-L1 adipocytes.

Although, in a previous report, stimulation of β_3 -ARs expressed in CHO/K1 cells failed to provide clear demonstration of p38 MAPK phosphorylation (Gerhardt *et al.*, 1999), such phosphorylation was evident in the present report using 3T3-L1 adipocytes. The most prominent difference between the cells used here and those used

previous is suggested to be the receptor-G protein coupling. The β_3 -AR coupled to Gi protein in the CHO/K1 cells (Gerhardt *et al.*, 1999), whereas it was suggested that β_3 -AR coupled functionally to Gs protein in 3T3-L1 adipocytes (Figure 4c,d). This difference in receptor-G protein coupling might bring about the difference in the ability to activate p38 MAPK. Very recently, during revising this manuscript, it was confirmed that a β_3 -AR agonist CL316,243 caused p38 MAPK phosphorylation in C3H10T1/2 and brown adipocytes in a cyclic AMP/PKA dependent pathway (Cao *et al.*, 2001). This report might strengthen the possibility that phosphorylation of p38 MAPK is determined by G protein(s) functionally coupling to β_3 -AR, although it was not confirmed which G protein was involved in the phosphorylation of p38 MAPK.

CTX causes activation of Gs protein by the ADP-ribosylation of Gs protein at specific amino acid residues,

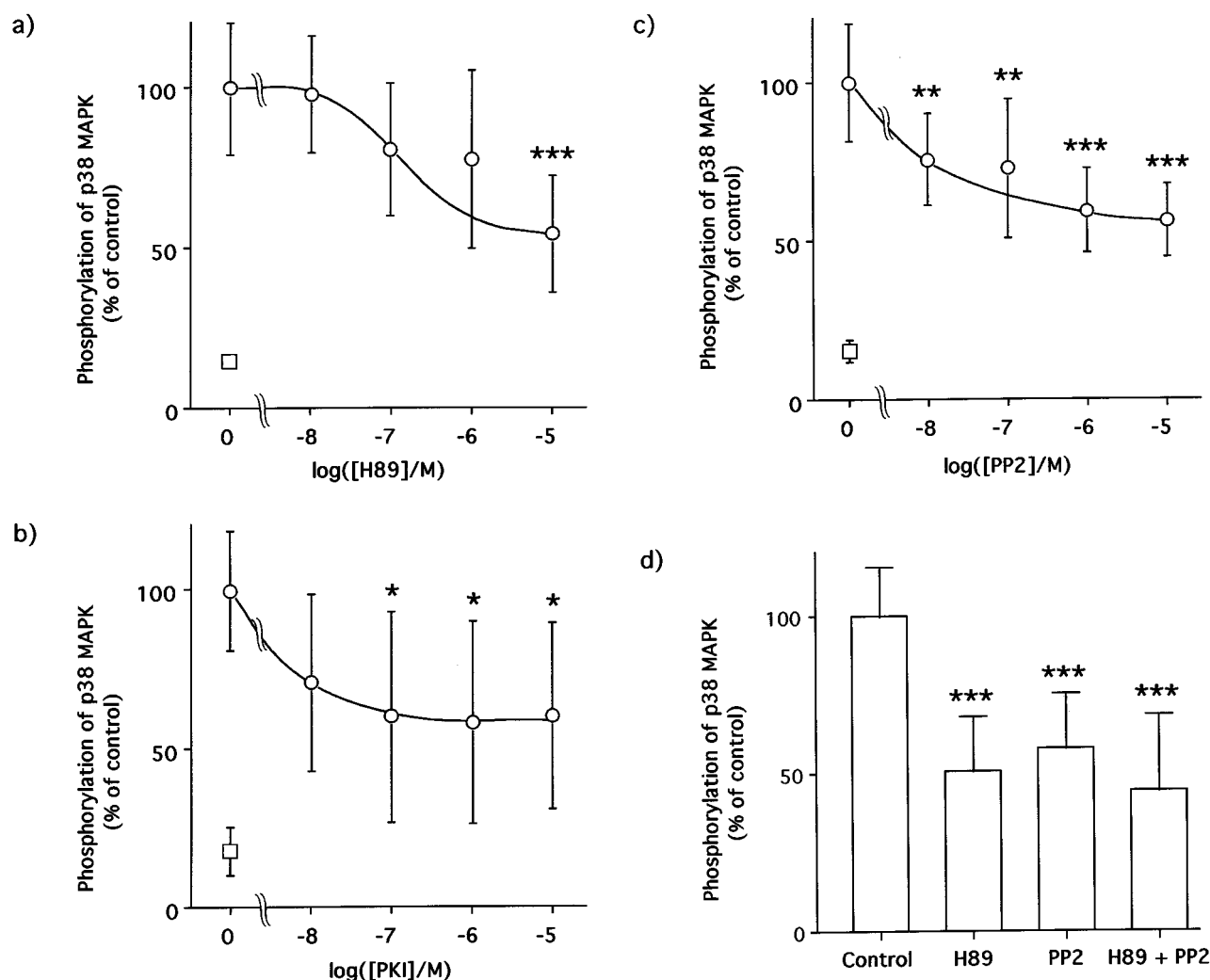


Figure 6 Effects of PKA and a src-family kinases inhibitors on p38 MAPK phosphorylation by BRL37344A in 3T3-L1 adipocytes. The adipocytes were treated with H89, PKI-(14–22)-amide and/or PP2 at the indicated concentrations for 30 min, and then stimulated with 10 nM BRL37344A for 30 min at 37°C. The degree of p38 MAPK phosphorylation was expressed as open circle and bars as a percentage of control that obtained without inhibitors (means \pm s.d. of four independent experiments). The open square expressed the basal value obtained without BRL37344A and inhibitors. The data in (a, b and c) were compared with the values obtained without inhibitors as controls by one-way ANOVA with Dunnett's multiple comparison (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). The values in (d) are significantly different from that obtained without inhibitors by one-way ANOVA and Tukey's multiple comparison (***: $P < 0.001$).

which leads to inhibition of GTP-hydrolyzing activity (Cassel & Selinger, 1997). As shown in Figure 5a,b, short-term treatment of 3T3-L1 adipocytes with CTX caused phosphorylation of p38 MAPK in a dose- and time-dependent manner. Forskolin is known as a direct activator of AC (Seamon & Daly, 1981) although it has the ability to inhibit AC under certain circumstances (Watanabe & Jakobs, 1986). Treatment of the 3T3-L1 adipocytes with forskolin also increased p38 MAPK phosphorylation (Figure 5c,d). These results indicate that activation of Gs protein and AC is sufficient to cause phosphorylation of p38 MAPK in 3T3-L1 adipocytes, and that PKA is likely to participate in the phosphorylation.

As shown in Figure 6a, the phosphorylation of p38 MAPK by BRL37344A (10 nM) was inhibited by a highly selective PKA inhibitor H89 in a dose-dependent manner and reached a maximal reduction of 50% at a concentration of 10 μ M, whereas ERK1/2 phosphorylations *via* β_3 -ARs were com-

pletely inhibited at the same concentration (Mizuno *et al.*, 1999). Although H89 was reported to inhibit ligand binding to β_1 and β_2 -ARs (Penn *et al.*, 1999), such an antagonism was not found in the case with β_3 -AR even at 50 μ M (Fredriksson *et al.*, 2001), suggesting that the effect of H89 to inhibit the p38 MAPK phosphorylation was brought about by the inhibition of PKA but not by the inhibition of binding of BRL37344A to β_3 -AR. To support this hypothesis, the effect of cell-permeable PKI-(14–22)-amide was examined, which was also known as an inhibitor for PKA (Glass *et al.*, 1989). PKI-(14–22)-amide decreased the phosphorylation of p38 MAPK by BRL37344A in a dose-dependent manner and the maximal effect was obtained at 10 μ M although the inhibition was about 50%. The dose-dependency was well consistent with the previous report showing that PKI-(14–22)-amide almost completely inhibited PKA activity at 10 μ M or higher concentrations (Glass *et al.*, 1989). These result might show

that the complete inhibition of PKA brought about almost 50% reduction of the p38 MAPK phosphorylation, in other words, PKA-dependent pathway(s) participate, at least to some degree, in β_3 -AR mediated p38 MAPK phosphorylations.

The participation of cAMP and PKA in p38 MAPK phosphorylation by a β_3 -AR agonist CL316,243 have already confirmed using C3H10T1/2 and brown adipocytes (Cao *et al.*, 2001). Treatment of the cells with the PKA inhibitor H89 completely abrogated the p38 MAPK phosphorylation in C3H10T1/2 and brown adipocytes, suggesting that the PKA-dependent pathway was the sole pathway leading to the p38 MAPK phosphorylation at least in C3H10T1/2 and brown adipocytes (Cao *et al.*, 2001). On the other hand, our result with 3T3-L1 adipocytes and two PKA inhibitors H89 and PKI-(14–22)-amide failed to show the obligatory role of PKA in p38 MAPK phosphorylation. These results suggested the possibility that other pathway(s) independent from PKA also play(s) a role in the phosphorylation of p38 MAPK *via* β_3 -AR.

Recent studies have shown that src-family tyrosine kinase(s) play(s) an important role not only in MAPK activation *via* various receptors but also in β_3 -AR signalling as a downstream element of PKA (Lindquist *et al.*, 2000) or by directly binding to β_3 -AR in a Gi-protein dependent manner (Cao *et al.*, 2000). To elucidate the role of src-family tyrosine kinase(s) in the p38 MAPK phosphorylation by BRL37344A, the effect of PP2, which selectively inhibits src-family tyrosine kinases (Hanke *et al.*, 1996), was examined. Treatment with PP2 markedly decreased p38 MAPK phosphorylation by BRL37344A in 3T3-L1 adipocytes in a dose-dependent manner and achieved almost a maximal

reduction of 50% at a concentration of 1.0 μ M as shown in Figure 6c. At this concentration, PP2 have reported to selectively inhibit src-family kinases (Hanke *et al.*, 1996). In addition, further inhibitions were not observed even at 10 times higher concentration (Figure 6c). The dose-dependency was well consistent with the previous report that examined the effect of PP2 to inhibit activity of a src-family tyrosine kinase lck (Hanke *et al.*, 1996). Taken together, the result strongly suggested that the reduction of p38 MAPK phosphorylation by the treatment with PP2 was due to inhibition of src-family kinase(s) although the inhibition by PP2 was 50% even at the maximal. Combined use of H89 and PP2 even at 10 μ M did not enhance the inhibition (Figure 6d), suggesting that PKA and src-family kinase(s) share the same pathway.

In conclusion, it was suggested that stimulation of β_3 -AR causes phosphorylation and activation of p38 MAPK and that these effects occur, at least in part, *via* a Gs-protein coupled pathway involving PKA and src-family tyrosine kinase(s), although the physiological significance of p38 MAPK activated *via* β_3 -ARs and the contribution of the unidentified pathway in the phosphorylation remain to be clarified.

We would like to acknowledge Dr Luciano Manara and Dr Martine Combes for kindly supplying us with the β_3 -AR antagonist SR59230A and β_3 -AR agonist SR58611A. This work was supported in part by a grant from The Smoking Research Foundation to Y.Watanabe.

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(Received April 26, 2001)

Revised October 26, 2001

Accepted November 29, 2001)