Proinsulin C-peptide rapidly stimulates mitogen-activated protein kinases in Swiss 3T3 fibroblasts: requirement of protein kinase C, phosphoinositide 3-kinase and pertussis toxin-sensitive G-protein

Takanori KITAMURA*, Kazuhiro KIMURA*¹, Bae Dong JUNG*, Kennedy MAKONDO*, Shiki OKAMOTO*, Xavier CAÑAS*, Naoki SAKANE†, Toshihide YOSHIDA† and Masayuki SAITO*

*Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan, and †First Department of Internal Medicine, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan

It has been demonstrated that proinsulin C-peptide possesses several biological activities and that its specific binding sites are present on the surface of cell membranes. However, the molecular and cellular mechanisms of C-peptide actions are poorly known. In the present study we examined the possible involvement of the mitogen-activated protein kinase (MAPK) pathway in C-peptide effects. C-peptide induced the phosphorylation of MAPK [p44 extracellular signal-regulated kinase 1 (ERK1) and p42 ERK2] in Swiss 3T3 and 3T3-F442A fibroblasts but not in 3T3-L1 fibroblasts and some other cell lines such as L₆E₉ muscle cells. In Swiss 3T3 cells, C-peptide-induced phosphorylation of MAPK was dependent on time and concentration, being maximal at 1 min and at 1 nM C-peptide and was accompanied by an increase in MAPK activity and MAPK kinase (MEK) phosphorylation. The MAPK phosphorylation by C-peptide was

abolished by treatment with pertussis toxin (PTX) and also with a MEK inhibitor, PD 98059. In addition, MAPK phosphorylation was attenuated by treatment with a phosphoinositide 3-kinase (PI-3K) inhibitor, wortmannin, and with a protein kinase C (PKC) inhibitor, GF109203X, and by down-regulation of PKC by prolonged treatment with PMA. Similar effects of the inhibitors and PTX were found on the MAPK phosphorylation induced by neuropeptide Y. These results suggest that C-peptide activates MAPK through a putative G_i/G_o-linked receptor for C-peptide and through PI-3K-dependent and PKC-dependent pathways.

Key words: 3T3-F442A, insulin, MAP kinase kinase, neuropeptide Y, wortmannin.

INTRODUCTION

C-peptide is a connecting segment of proinsulin and is secreted from pancreatic β -cells into the circulation along with insulin after the cleavage of proinsulin. Although C-peptide was formerly considered biologically inactive, more recent studies have suggested that it is a biologically active peptide and might be involved in the development of diabetic microangiopathy and neuropathy [1,2]. Administration of C-peptide to insulin-dependent diabetic patients or diabetic animals has been shown to decrease glomerular hyperfiltration [3–5], to diminish the leakage of albumin or fluorescein across the blood–retina barrier [3,6], to increase glucose uptake in skeletal muscle [7,8] and to improve autonomic nerve and microvascular functions [3,5,6,8–11].

The presence of a putative receptor for C-peptide had not been proved by the conventional method with iodinated C-peptide in various cell preparations, except in insulin-secreting β -cells [7,12,13]. However, Rigler et al. [13] have recently demonstrated the presence of specific binding sites for C-peptide on the surface of human renal tubular cells, endothelial cells and fibroblasts by using fluorescence correlation spectroscopy. These binding sites can be displaced with a stereospecific form of C-peptide but not with insulin, proinsulin or insulin-like growth factor I or II [13]. It has also been demonstrated that C-peptide increases Na⁺,K⁺-ATPase activity in rat renal tubule segments and that the effect of C-peptide is abolished by either pertussis toxin (PTX) or a

protein phosphatase 2B (calcineurin) inhibitor [14]. Thus it seems possible that C-peptide exhibits its activities through a specific PTX-sensitive G-protein-coupled receptor for C-peptide and the activation of a Ca²⁺-dependent intracellular signalling pathway.

Ohtomo et al. [14] reported that the C-peptide-induced activation of Na⁺,K⁺-ATPase is markedly enhanced in the presence of subthreshold concentrations of neuropeptide Y (NPY). NPY has recently been shown to stimulate mitogen-activated protein kinase (MAPK) via the Y1- or Y5-NPY receptors [15,16]. MAPKs, also known as extracellular signal-regulated kinase (ERK), are a family of protein-serine/threonine kinases, and are stimulated by a variety of ligands through their receptors, including receptor tyrosine kinases and G-protein-coupled receptors [17–19]. Based on the fact that C-peptide increases Na⁺,K⁺-ATPase activity, which is abolished by PTX treatment and enhanced by NPY [14], and that NPY stimulates MAPK [15,16], thus we assumed that C-peptide might activate MAPK through a putative PTX-sensitive G-protein-coupled receptor.

The aim of the present study was to examine the effect of C-peptide on MAPK activation as judged by specific phosphorylation (Thr-202/Tyr-204) of p44 and p42 MAPK (ERK1 and ERK2 respectively). Because a limited number of cells are known to respond to C-peptide, we first tested MAPK phosphorylation by C-peptide in various cell lines, then examined the general characteristics of the reaction and the effect of

Abbreviations used: EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; NPY, neuropeptide Y; PDGF, platelet-derived growth factor; PI-3K, phosphoinositide 3-kinase; PKC, protein kinase C; PTX, pertussis toxin.

¹ To whom correspondence should be addressed (e-mail k-kimura@vetmed.hokudai.ac.jp).

various inhibitors of intracellular signalling pathways on the C-peptide-induced phosphorylation, comparing them with those on NPY-induced MAPK phosphorylation of NPY.

EXPERIMENTAL

Materials

Human C-peptide was generously provided by Eli Lilly Co. (Indianapolis, IN, U.S.A.). Reverse-sequence (retro-sequenced) or all-D-amino acid human C-peptides were obtained from Bio-Synthesis (Lewisville, TX, U.S.A.). Rat C-peptides 1 and 2 were bought from Yanaihara Institute (Shizuoka, Japan). Bovine insulin, mouse epidermal growth factor (EGF) and human recombinant platelet-derived growth factor-AB (PDGF) were from Sigma Chemical Co. (St Louis, MO, U.S.A.). NPY and bombesin were purchased from Peptide Institute (Osaka, Japan) and American Peptide Co. (Sunnyvale, CA, U.S.A.) respectively.

Cell culture

The following cell lines were used: Swiss 3T3, a mouse embryonic fibroblast line; 3T3-L1 and 3T3-F442A, cloned cell lines from Swiss 3T3 cell that can differentiate to adipose cells [20]; L₆E₉, rat muscle cell; HepG2, a human hepatocellular carcinoma-derived cell line; C6, a rat glioma-derived cell line; NG108.15, a hybridoma between C6BU-1 and neuroblastoma N18TG-2; GH₄C₁, rat somatomammotrophic cell. These cell lines, except GH₄C₁, were individually cultured in 100 mm dishes containing Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD, U.S.A.) supplemented with 10% (v/v) fetal bovine serum in a humidified air/CO₂ (19:1) atmosphere at 37 °C. GH₄C₁ cells were cultured in Ham's F-12 nutrient mixture (Gibco-BRL) with 10% (v/v) FBS.

Treatment of cells

After cells had been grown to confluence, they were cultured with a serum-free medium for 2 h and kept in serum-free Hanks buffer. The cells were then treated at 37 °C with either human C-peptide or other peptides, all of which were dissolved in PBS, for the durations indicated in the Figure legends. Subsequently, the cells were washed twice with Hanks buffer and lysed with 1 ml of ice-cold lysis buffer [50 mM Hepes (pH 7.5)/150 mM NaCl/5 mM EDTA/10 mM sodium pyrophosphate/2 mM NaVO₃ containing protease mixture (Complete™; Boehringer Mannheim, GmbH, Germany) and 1% (v/v) Nonidet P40]. The lysate was kept on ice for 30 min and centrifuged at 15000 g for 20 min at 4 °C. The supernatant was removed and stored at -80 °C. Proteins were determined by the method of Lowry et al. [21] with BSA as a standard.

Treatment of cells with PTX (100 ng/ml; List Biological Laboratories, Campbell, CA, U.S.A.) or PMA (100 nM; Sigma) was performed for 22 h in fetal-bovine-serum-containing medium and for 2 h in a serum-free medium and Hanks buffer. Treatment of cells with 20 µM PD 98059 [a MAPK kinase (MEK) inhibitor (Biomol Research Laboratories, Plymouth Meeting, PA, U.S.A.)] was performed for 2 h in a serum-free medium and Hanks buffer. The treated cells were then stimulated by C-peptide or other peptides. Wortmannin (100 nM) [a phosphoinositide 3-kinase (PI-3K) inhibitor; a gift from Dr Satoshi Nakanishi (Kyowa Hakko Kogyo Co., Ltd., Shizuoka, Japan)] or 5 µM GF109203X [a protein kinase C (PKC) inhibitor (CalBiochem-Novabiochem Ltd., Nottingham, U.K.)] was added to serum-free Hanks buffer; cells were stimulated 10 min later.

Western blot analysis

Aliquots of the supernatant (25 µg of protein) were separated by SDS/PAGE and transferred on PVDF membranes (Immobilon™; Millipore, Bedford, MA, U.S.A.). The membranes were incubated first in a blocking buffer [20 mM Tris/HCl (pH 7.5)/150 mM NaCl] containing 0.1% Tween 20 and 5% (v/v) skimmed milk, then in a buffer containing an antibody against either phospho-specific p44/42 MAPK (Thr-202/Tyr-204) [NEB (New England Biolabs), Beverly, MA, U.S.A.], p44/42 MAPK (NEB), phospho-specific MEK1/2 (Ser-217/Ser-221) (NEB) or MEK1/2 (NEB). The bound antibody was detected with horseradish peroxidase-linked goat anti-rabbit immunoglobulin (Zymed Laboratories, South San Francisco, CA, U.S.A.) and an enhanced chemiluminescence system (Amersham, Little Chalfont, Bucks., U.K.). The intensity of chemiluminescence for the corresponding proteins was analysed by using NIH Image, a public-domain image processing and analysis program.

MAPK assay

The MAPK activity was measured with a p44/42 MAPK assay kit (NEB), in accordance with the manufacturer's recommended protocols. In brief, Swiss 3T3 cells were treated with either PBS or 1 nM human C-peptide for 3 min, washed with ice-cold PBS and lysed with 1 ml of a lysis buffer [20 mM Tris/HCl (pH 7.5)/150 mM NaCl/1 mM EDTA/1 mM EGTA/1 mM β-glycerophosphate/2.5 mM sodium pyrophosphate/1 mM NaVO₃ containing protease mixture (Complete™) and 1% (v/v) Triton X-100]. After centrifugation for 20 min at 4 °C, the supernatant was subjected to immunoprecipitation with phospho-specific p44/42 MAPK (Tyr-204) monoclonal antibody and Protein A-Sepharose CL-4B [50% (w/v) slurry] (Pharmacia Biotech, Uppsala, Sweden). After two washes with ice-cold lysis buffer and two washes with ice-cold kinase buffer [25 mM Tris/HCl (pH 7.5)/5 mM β-glycerophosphate/2 mM dithiothreitol/0.1 mM NaVO₃/10 mM MgCl₂], the pellets were incubated for 30 min at 30 °C with the kinase buffer containing 200 µM ATP and 2 µg of Elk-1 fusion protein. The reaction was stopped by the addition of SDS gel-loading buffer; the samples were then subjected to SDS/PAGE followed by Western blot analysis with a phospho-specific Elk-1 (Ser-383) antibody and a horseradish peroxidase-conjugated F(ab')₂ fragment of goat anti-rabbit immunoglobulin. MAPK activities were determined by scanning the X-ray film as described above.

Statistical analysis

Results are expressed as means ± S.E.M. and analysed by analysis of variance followed by Fisher's protected least-squares difference or Student's *t* test. *P* < 0.05 was considered to be statistically significant.

RESULTS

The MAPK cascade is known to be activated in response to mitogenic stimuli in a wide variety of cell types. For example, insulin is a representative stimulant in fibroblast-derived cell lines such as Swiss 3T3 [22]. To examine whether such cells also respond to C-peptide, several cell lines were incubated with C-peptide and the phosphorylation of MAPK was examined. As shown in Figure 1, C-peptide (1 nM) induced the phosphoryl-

Figure 1 Effects of C-peptide on phosphorylation of p44/42 MAPK in various cell lines

Swiss 3T3, 3T3-L1, 3T3-F442A, HepG2, L₆E₉, C6, GH₄C₁ and NG108.15 cells were treated with (+) or without (–) human C-peptide (1 nM) for 3 min. The lysate was subjected to analysis with antibodies against phospho-specific p44/42 MAPK (phosphorylated MAPK) and p44/42 MAPK (total MAPK). Representative results from three independent experiments are shown.

ation of MAPK in Swiss 3T3 cells, without noticeable changes in the total amount of MAPK. Similarly, C-peptide caused the phosphorylation of MAPK in 3T3-F442A cells. In contrast, C-peptide had no apparent effect on MAPK in 3T3-L1 fibroblasts, L₆E₉ muscle cells, HepG2 hepatoma cells, C6 glioma cells, NG108.15 neuroblastoma cells and GH₄C₁ somatomammotrophic cells (Figure 1). Similarly to human C-peptide, rat C-peptides 1 and 2 were also effective in inducing MAPK phosphorylation in Swiss 3T3 (Figure 2) and 3T3-F442A cells but not in 3T3-L1 cells (results not shown). However, neither retro-sequenced nor all-D-amino acid human C-peptide stimulated the phosphorylation of MAPK in Swiss 3T3 cells (Figure 2).

Both 3T3-L1 and 3T3-F442A cells are derived from Swiss 3T3 cells, fibroblast-like in shape and capable of differentiating into adipocytes on stimulation by dexamethasone and 3-isobutyl-1-methylxanthine. However, in 3T3-L1 cells C-peptide did not affect the phosphorylation of MAPK (Figure 1). The different responses to C-peptide in these cell lines might be due to the fact that the basal level of phosphorylation of MAPK was much higher in 3T3-L1 cells; that is, MAPK might be maximally phosphorylated without stimulation. To test this possibility, the stimulatory effect of C-peptide was compared with those of insulin. In Swiss 3T3 and 3T3-F442A cells, both C-peptide and insulin induced the phosphorylation of MAPK, although the magnitude of the response was different (Figure 3). In 3T3-L1 cells, insulin significantly enhanced the phosphorylation of MAPK, whereas C-peptide did not (Figure 3). These results thus confirmed the unresponsiveness of 3T3-L1 cells to C-peptide.

We next determined concentration-dependent and time-dependent effects of C-peptide on the phosphorylation of MAPK in Swiss 3T3 cells. Phosphorylation of MAPK seemed to increase at C-peptide concentrations of as low as 1 pM and reached maximal levels at 1 nM (Figure 4, left panels). The half-maximal effective dose was 0.25 ± 0.05 nM for p44 MAPK and 0.28 ± 0.09 nM for p42 MAPK. A higher concentration of C-peptide (100 nM) was not effective in the phosphorylation of MAPK. The total amount of MAPK was not changed at any concentration of C-peptide tested. MAPK phosphorylation was examined at various times after the addition of C-peptide (1 nM). As shown in Figure 4 (right panels), the phosphorylation was quickly induced, reaching a maximum at 1 min, and gradually decreased to the basal level. To confirm that the site-specific phosphorylation of MAPK activates the enzyme, the kinase activity of MAPK was determined. The MAPK activity was

Figure 2 Effect of various types of C-peptide on phosphorylation of p44/42 MAPK in Swiss 3T3 cells

Swiss 3T3 were treated with either PBS (control) or 1 nM human C-peptide (L-AA), rat C-peptide 1 (rat1), rat C-peptide 2 (rat2), all-D-amino acids human C-peptide (D-AA) and retro-sequenced human C-peptide (retro) for 3 min. The lysate was subjected to analysis with antibodies against phospho-specific p44/42 MAPK (phosphorylated MAPK) and p44/42 MAPK (total MAPK). Representative results from three independent experiments are shown.

Figure 3 Quantitative changes in phosphorylated p44/42 MAPK induced by insulin and C-peptide in Swiss 3T3, 3T3-L1 and 3T3-F442A cells

Swiss 3T3 ($n = 3$), 3T3-L1 ($n = 5$) and 3T3-F442A ($n = 3$) cells were treated with either human C-peptide (1 nM) or bovine insulin (100 nM) for 3 min. The untreated cells were used as a control. The ratio of phosphorylated MAPK (p44 or p42) to the total amount of respective MAPK was calculated and expressed relative to that in control cells. * $P < 0.05$ compared with controls.

increased 2.4 ± 0.2 -fold after stimulation for 3 min with C-peptide (1 nM) compared with those treated with PBS ($P < 0.05$).

In Swiss 3T3 cells, MAPK is known to be activated not only by insulin but also by some peptides such as bombesin [23,24], EGF [23] and PDGF [25]. In addition, MAPK is activated by NPY in erythroleukaemia cells [15] and primary cardiomyocytes [16]. Considering the proposed interaction between the effects of NPY and C-peptide on Na⁺,K⁺-ATPase, we first examined whether MAPK is activated by NPY in Swiss 3T3 cells too. A 3 min treatment with 100 nM NPY produced a 2–6-fold increase in the phosphorylation of p44 and p42 MAPK (Figures 5–8).

There have been several reports on the signal transduction pathways for MAPK activation in Swiss 3T3 cells; the possible involvement of PTX-sensitive G-protein, PKC and PI-3K has been suggested. We therefore examined the effects of various inhibitors of the signal-transducing components on C-peptide-induced MAPK activation, comparing them with those on the

Figure 4 Dose- and time-dependent response to C-peptide of p44/42 MAPK phosphorylation in Swiss 3T3 cells

Swiss 3T3 cells were treated with increasing concentrations of C-peptide for 3 min (left panels) or 1 nM C-peptide (right panels) for 1–60 min. The amounts of phosphorylated p44 MAPK (●), phosphorylated p42 MAPK (○), total p44 MAPK (▲) and total p42 MAPK (△) are expressed relative to those of untreated control cells. Results are means $\pm$ S.E.M. for four independent experiments. * $P < 0.05$ compared with controls. Representative results of Western blot analysis are also shown.

activation induced by NPY and other peptides. First we investigated the effects of C-peptide and NPY on the phosphorylation of MEK, an enzyme that phosphorylates and activates MAPK. As shown in Figure 5 (upper panels), both C-peptide and NPY increased the site-specific phosphorylation of MEK, leading to an increase in MEK activity. In the presence of PD 98059, a MEK inhibitor, neither C-peptide nor NPY induced MAPK phosphorylation (Figure 5, lower panels).

Next we determined a role of G-protein by using PTX, a specific inhibitor of G_i/G_o proteins. Previous treatment of the cells with PTX did not affect the basal levels of MAPK phosphorylation but blocked those induced by C-peptide (Figure 6). Similarly, NPY failed to stimulate MAPK phosphorylation in the cells treated with PTX. In contrast, bombesin (10 nM) stimulated the phosphorylation even after treatment with PTX, in accordance with previous reports [23,24].

Wortmannin, a PI-3K inhibitor, also prevented both C-peptide- and NPY-induced MAPK phosphorylation but not PDGF-induced phosphorylation (Figure 7). Similarly, GF109203X, a PKC inhibitor, completely abolished the phosphorylation induced both by C-peptide and by NPY (Figure 8, left panels). A role of PKC was confirmed further by the findings that C-peptide and NPY failed to stimulate MAPK phosphorylation when PKC was down-regulated by pretreatment of the cells with PMA for 24 h (Figure 8, right panels). In contrast, treatment with PMA did not affect the EGF-induced MAPK phosphorylation.

DISCUSSION

In the present study we have demonstrated for the first time that proinsulin C-peptide stimulated the Thr-202/Tyr-204 phosphorylation of p44/p42 MAPK. Maximal phosphorylation occurred within 1 min and at 1 nM C-peptide. The effective

concentrations of C-peptide were comparable with physiological levels (0.1–1.0 nM) of C-peptide in the circulation [26–28], and also with the association constant (K_{ass}) value (2.0–3.3 nM) obtained by a binding study *in vitro* [13]. Because MAPK phosphorylation by C-peptide accompanied comparable increases in the catalytic activity of MAPK and the activation of MEK, our results therefore suggest that C-peptide can activate the MAPK cascade under physiological conditions.

C-peptide stimulated the phosphorylation of MAPK only in Swiss 3T3 and 3T3-F442A cells, not in the other cell lines tested. It is particularly interesting that C-peptide did not induce MAPK phosphorylation in 3T3-L1 cells, which were originally derived from Swiss 3T3 cells. Because insulin stimulated the MAPK cascade in all three cell lines, the unresponsiveness of 3T3-L1 cells to C-peptide might not be due to differences in the MAPK cascade itself. The magnitude of the response to C-peptide considerably varied in individual experiments, even in Swiss 3T3 cells (Figures 1–8). In preliminary experiments we found that the responses tended to be smaller in the cells that were cultured for longer periods with repeated passages. This might be related to the unresponsiveness to C-peptide in 3T3-L1 cells.

The responses specific to limited cell lines, together with time- and dose-dependent responses, imply that a receptor for C-peptide is present on the surface of Swiss 3T3 and 3T3-F442A cells. Moreover, the stimulatory effect of C-peptide was diminished when the cells were treated with PTX. These results are consistent with the abolition by PTX of the binding of C-peptide to the cell membrane [13] and of the C-peptide-induced activation of Na^+, K^+ -ATPase in renal tubules [14], suggesting that the receptor is a G-protein-coupled type. In the present study we found that NPY and bombesin also stimulated MAPK phosphorylation in Swiss 3T3 cells. Treatment with PTX inhibited the stimulatory effect of NPY, which is known to be mediated through a G_i -coupled NPY receptor, whereas it did not

Figure 5 Involvement of MEK in C-peptide-induced p44/42 MAPK phosphorylation in Swiss 3T3 cells

Upper panels: Swiss 3T3 cells were treated with C-peptide (1 nM) or NPY (100 nM) for 3 min. The amounts of phosphorylated MEK and total MEK are expressed relative to those of untreated control cells. Representative results of Western blot analysis are also shown. Lower panels: Swiss 3T3 cells were treated with PD 98059 (20 μ M) for 2 h and then stimulated with C-peptide or NPY for 3 min. Cells without inhibitor treatment were used as a control. The amounts of phosphorylated MAPK and total MAPK are expressed relative to those of control cells. Results are means $\pm$ S.E.M. for five independent experiments. * P < 0.05 compared with controls; † P < 0.05 compared with no inhibitor treatment.

affect those of bombesin, which are mediated through a G_q/G_{11} -coupled bombesin receptor (Figure 6) [16,24]. It is therefore likely that the putative receptor for C-peptide might be a PTX-sensitive G-protein (G_i/G_o)-coupled receptor.

At present we have little information about the putative receptor for C-peptide, which might be similar to or identical with that reported by Rigler et al. [13], who detected the binding of C-peptide to cell membrane by the fluorescence correlation spectroscopy method. They showed that the binding was displaced with unlabelled native C-peptide (L-form), but not with all-D-amino acid C-peptide [13]. In line with their results, we found that native C-peptide, but not all-D-form C-peptide, stimulated MAPK phosphorylation. Furthermore, rat C-peptides 1 and 2, which have nine amino acid substitutions with regard to human C-peptide, were also effective in MAPK phosphorylation, suggesting that these substitutions might not alter the structural features of C-peptide. Ido et al. [6] reported that D-form and retro-sequenced C-peptides, as well as L-form human and rat C-peptides, improved hyperglycaemia-induced vascular dysfunction, whereas the both peptides failed to stimulate MAPK phosphorylation. The reason for this apparent discrepancy in the effect of D-form and retro-sequenced C-

Figure 6 Effects of PTX on C-peptide-induced p44/42 MAPK phosphorylation in Swiss 3T3 cells

Swiss 3T3 cells were treated with 100 ng/ml PTX for 24 h, then stimulated with C-peptide (1 nM), NPY (100 nM) or bombesin (10 nM) for 3 min. Cells without PTX treatment were used as a control. The amounts of phosphorylated MAPK and total MAPK are expressed relative to those of control cells. Results are means $\pm$ S.E.M. for three to five independent experiments. * P < 0.05 compared with controls; † P < 0.05 compared with no PTX treatment.

Figure 7 Effects of wortmannin on C-peptide-induced p44/42 MAPK phosphorylation in Swiss 3T3 cells

Swiss 3T3 cells were treated with a PI-3K inhibitor, wortmannin (100 nM), for 10 min, then stimulated with C-peptide (1 nM), NPY (100 nM) or PDGF (100 nM) for 3 min. Cells without inhibitor treatment were used as a control. The amounts of phosphorylated MAPK and total MAPK are expressed relative to those of control cells. Results are means $\pm$ S.E.M. for five independent experiments. * P < 0.05 compared with controls; † P < 0.05 compared with no inhibitor treatment.

peptides is not yet clear. To clarify the molecular structure of the putative C-peptide receptor, further studies such as cDNA cloning are needed. MAPK activation would be a functional marker for molecular cloning of the receptor.

The receptors and post-receptor pathways to MAPK have been studied extensively in various tissues and cell lines [17–19]. In Swiss 3T3 cells, bombesin-induced p42 MAPK activation is mediated predominantly by PKC but independently of a G_i

Figure 8 Effects of prolonged treatment with PMA and PKC inhibitor on C-peptide-induced p44/42 MAPK phosphorylation in Swiss 3T3 cells

Left panels: Swiss 3T3 cells were treated with GF109203X, a PKC inhibitor (5 μ M), for 10 min and stimulated with C-peptide (1 nM) or NPY (100 nM). Right panels: Swiss 3T3 cells were treated with PMA (100 nM) for 24 h, then stimulated with C-peptide (1 nM), NPY (100 nM) or EGF (100 nM). Cells without PMA treatment were used as a control. The amounts of phosphorylated MAPK and total MAPK are expressed relative to those of control cells. Results are means $\pm$ S.E.M. for five independent experiments. * P < 0.05 compared with controls; † P < 0.05 compared with no PMA treatment.

signalling pathway [23,24]. EGF and PDGF (100 nM) act on their tyrosine kinase type-receptors and activate p21^{ras} and a kinase cascade comprising raf-1, MEK and MAPK [17–19], which is independent of PKC and PI-3K respectively [23,25]. These were also confirmed in the present study by using some inhibitors of PI-3K (wortmannin) and PKC (GF109203X). In contrast, we found that the C-peptide-induced MAPK phosphorylation was diminished after treatments not only with PTX but also with the inhibitors of PI-3K and PKC. Thus the pathway of C-peptide-induced MAPK activation might be rather different from those used by bombesin, PDGF and EGF. However, quite similar effects of PTX, wortmannin, GF109203X and down-regulation of PKC were found in MAPK phosphorylation induced by C-peptide and NPY. It therefore seems possible that the two peptides share a common activation pathway involving PKC and PI-3K.

The physiological relevance of MAPK activation by C-peptide is not clear at present, although MAPK is known to be important in the mitogenic signalling of a variety of polypeptide growth factors. Hehenberger et al. [29] have reported that C-peptide at a supraphysiological concentration (70 nM) stimulated the proliferation of fibroblasts derived from patients with insulin-dependent diabetes mellitus, and that the effect of C-peptide was partly prevented by an inhibitor of MEK. Their findings seem compatible with our present results, suggesting again that the MAPK cascade is involved in the action of C-peptide. However, we could not find any proliferative response to C-peptide in Swiss 3T3 cells (T. Kitamura, K. Kimura and M. Saito, unpublished work). Thus the precise roles of the activation of MAPK by C-peptide, and particularly its relation to the effect of C-peptide on renal and vascular functions, remain to be elucidated.

In summary, proinsulin C-peptide stimulates MAPK in fibroblasts through a common or similar pathway to that for NPY, which requires PKC, PI-3K and PTX-sensitive G-protein.

This work was supported in part by PROBRAIN from the Bio-oriented Technology Research Advancement Institution, Japan. X.C. is the recipient of a European Commission fellowship (DG XII-STF).

REFERENCES

- Wahren, J. and Johansson, B. L. (1998) New aspects of C-peptide physiology. *Horm. Metab. Res.* **30**, A2–A5.
- Wahren, J., Johansson, B. L. and Wallberg-Henriksson, H. (1994) Does C-peptide have a physiological role? *Diabetologia* **37** (Suppl. 2), S99–S107.
- Johansson, B. L., Kernell, A., Sjöberg, S. and Wahren, J. (1993) Influence of combined C-peptide and insulin administration on renal function and metabolic control in diabetes type 1. *J. Clin. Endocrinol. Metab.* **77**, 976–981.
- Sjöquist, M., Huang, W. and Johansson, B. L. (1998) Effects of C-peptide on renal function at the early stage of experimental diabetes. *Kidney Int.* **54**, 758–764.
- Johansson, B. L., Sjöberg, S. and Wahren, J. (1992) The influence of human C-peptide on renal function and glucose utilization in type 1 (insulin-dependent) diabetic patients. *Diabetologia* **35**, 121–128.
- Ido, Y., Vindigni, A., Chang, K., Stramm, L., Chance, R., Heath, W. F., DiMarchi, R. D., Di Cera, E. and Williamson, J. R. (1997) Prevention of vascular and neural dysfunction in diabetic rats by C-peptide. *Science* **277**, 563–566.
- Zierath, J. R., Handberg, A., Tally, M. and Wallberg-Henriksson, H. (1996) C-peptide stimulates glucose transport in isolated human skeletal muscle independent of insulin receptor and tyrosine kinase activation. *Diabetologia* **39**, 306–313.
- Johansson, B. L., Linde, B. and Wahren, J. (1992) Effects of C-peptide on blood flow, capillary diffusion capacity and glucose utilization in the exercising forearm of type 1 (insulin-dependent) diabetic patients. *Diabetologia* **35**, 1151–1158.
- Johansson, B. L., Borg, K., Fernqvist-Forbes, E., Obergren, T., Remahl, S. and Wahren, J. (1996) C-peptide improves autonomic nerve function in IDDM patients. *Diabetologia* **39**, 687–695.
- Forst, T., Kunt, T., Pohlmann, T., Goitom, K., Engelbach, M., Beyer, J. and Pfützner, A. (1998) Biological activity of C-peptide on the skin microcirculation in patients with insulin-dependent diabetes mellitus. *J. Clin. Invest.* **101**, 2036–2041.
- Lindstrom, K., Johansson, C., Johnsson, E. and Haraldsson, B. (1996) Acute effects of C-peptide on the microvasculature of isolated perfused skeletal muscles and kidneys in rat. *Acta Physiol. Scand.* **156**, 19–25.
- Flatt, P. R., Swanston-Flatt, S. K., Hampton, S. M., Bailey, C. J. and Marks, V. (1986) Specific binding of the C-peptide of proinsulin to cultured β -cells from a transplantable rat islet cell tumor. *Biosci. Reports* **6**, 193–199.
- Rigler, R., Pramanik, A., Jonasson, P., Kratz, G., Jansson, O. T., Nygren, P.-A., Stahl, S., Ekberg, K., Johansson, B. L., Uhlen, S. et al. (1999) Specific binding of proinsulin C-peptide to human cell membranes. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 13318–13323.
- Ohtomo, Y., Aperia, A., Sahlgren, B., Johansson, B. L. and Wahren, J. (1996) C-peptide stimulates rat renal tubular Na^+/K^+ -ATPase activity in synergism with neurotensin. *Diabetologia* **39**, 199–205.
- Keffel, S., Schmidt, M., Bischoff, A. and Michel, M. C. (1999) Neuropeptide-Y stimulation of extracellular signal-regulated kinases in human erythroleukemia cells. *J. Pharmacol. Exp. Ther.* **291**, 1172–1178.

- 16 Pellieux, C., Sauthier, T., Domenighetti, A., Marsh, D. J., Palmiter, R. D., Brunner, H.-R. and Pedrazzini, T. (2000) Neuropeptide Y (NPY) potentiates phenylephrine-induced mitogen-activated protein kinase activation in primary cardiomyocytes via NPY Y5 receptors. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 1595–1600
- 17 Lopez-Illasaca, M. (1998) Signaling from G-protein-coupled receptors to mitogen-activated protein (MAP)-kinase cascades. *Biochem. Pharmacol.* **56**, 269–277
- 18 English, J., Pearson, G., Wilsbacher, J., Swantek, J., Karandikar, M., Xu, S. and Cobb, M. H. (1999) New insights into the control of MAP kinase pathways. *Exp. Cell Res.* **253**, 255–270
- 19 Marshall, C. J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**, 179–185
- 20 Green, H. and Kehinde, O. (1976) Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. *Cell* **7**, 105–113
- 21 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
- 22 Hansson, A. and Thoren, M. (1995) Activation of MAP kinase in Swiss 3T3 fibroblasts by insulin-like growth factor-I. *Growth Regul.* **5**, 92–100
- 23 Pang, L., Decker, S. J. and Saltiel, A. R. (1993) Bombesin and epidermal growth factor stimulate the mitogen-activated protein kinase through different pathways in Swiss 3T3 cells. *Biochem. J.* **289**, 283–287
- 24 Seufferlein, T. and Rozengurt, E. (1995) Sphingosylphosphorylcholine activation of mitogen-activated protein kinase in Swiss 3T3 cells requires protein kinase C and a pertussis toxin-sensitive G protein. *J. Biol. Chem.* **270**, 24334–24342
- 25 Duckworth, B. C. and Cantley, L. C. (1997) Conditional inhibition of the mitogen-activated protein kinase cascade by wortmannin. Dependence on signal strength. *J. Biol. Chem.* **272**, 27665–27670
- 26 Dreyer, M., Matthaei, S., Kuhnau, J. and Rudiger, H. W. (1986) Prolonged plasma half-life of insulin in patients with a genetic defect of high affinity binding sites. *Horm. Metab. Res.* **18**, 247–249
- 27 Horwitz, D. L., Starr, J. I., Mako, M. E., Blackard, W. G. and Rubenstein, A. H. (1975) Proinsulin, insulin, and C-peptide concentrations in human portal and peripheral blood. *J. Clin. Invest.* **55**, 1278–1283
- 28 Faber, O. K., Hagen, C., Binder, C., Markussen, J., Naithani, V. K., Blix, P. M., Kuzuya, H., Horwitz, D. L., Rubenstein, A. H. and Rossing, N. (1978) Kinetics of human connecting peptide in normal and diabetic subjects. *J. Clin. Invest.* **62**, 197–203
- 29 Hehenberger, K., Brismer, K., Kratz, G. and Johanson, B. (1997) C-peptide increases proliferation in fibroblasts derived from IDDM patients (abstract). *Eur. J. Endocrinol.* **136** (Suppl. 1), 15

Received 16 August 2000/2 January 2001; accepted 29 January 2001