

Stimulation of catecholamine biosynthesis via the PKC pathway by prolactin-releasing peptide in PC12 rat pheochromocytoma cells

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Abstract

We have previously shown that prolactin-releasing peptide (PrRP) stimulates catecholamine release from PC12 cells (rat pheochromocytoma cell line). However, it is not known whether PrRP also affects catecholamine biosynthesis. Thus, we examined the effect of PrRP on catecholamine biosynthesis in PC12 cells. PrRP31 (>10 nM) and PrRP20 (>100 nM) significantly increased the activity and expression level of tyrosine hydroxylase (TH), a rate-limiting enzyme, in catecholamine biosynthesis. However, the PrRP20-stimulated TH activity was markedly weaker than that of PrRP31. PrRP31 (>1 nM) and PrRP20 (>10 nM) significantly induced an increase in the level of PKC activity. Both Ro 32-0432 (a protein kinase C inhibitor) and H89 (a protein kinase A inhibitor)

effectively suppressed the PrRP31 (100 nM)-induced TH mRNA level. Next, we examined the effect of PrRP on mitogen-activated protein kinases (MAPKs). PrRP31 (1 µM) significantly induced an increase in the activity of extracellular signal-related kinases (ERKs) and the stress-activated protein kinase/c-jun N terminal kinase (SAPK/JNK). In contrast to ERKs and JNK, PrRP31 did not affect P38 MAPK activity. Consistent with these findings, pretreatment of cells with the MEK-1-inhibitor, PD-98059 (50 µM), significantly inhibited the PrRP31 (100 nM)-induced increase in TH mRNA. These results indicate that PrRP stimulates catecholamine synthesis through both the PKC and PKA pathways in PC12 cells.

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Introduction

Prolactin-releasing peptide (PrRP) is a novel hypothalamic hormone, initially identified by Hinuma *et al.* (1998) by the method of 'reverse' pharmacology. A 31-amino-acid peptide (PrRP31), with no significant homology to any known hormone, was isolated that had prolactin-releasing properties. Post-translational modification of the same gene product can give rise to another form of PrRP, named PrRP20 (Hinuma *et al.* 1998).

More recently, PrRP and its receptors have been reported to be expressed in numerous areas of the central nervous system (CNS), such as the hypothalamus and brainstem. Interestingly, PrRP has been colocalized with tyrosine hydroxylase (TH)-expressing medullary A1 and A2 neurons, suggesting that the peptide may have functions other than acting as a prolactin-releasing factor, such as modulating catecholaminergic reactions (Marchese *et al.* 1995, Welch *et al.* 1995, Iijima *et al.* 1999, Fujii *et al.* 1999, Kimura *et al.* 2000, Langmead *et al.* 2000).

PrRP and its receptor are highly expressed in the adrenal medulla as well as human pheochromocytomas, suggesting that these peptides may affect the function of

chromaffin cells in an autocrine and/or paracrine fashion (Roland *et al.* 1999, Chen *et al.* 1999, Nieminen *et al.* 2000, Takahashi *et al.* 2002). Very recently, PrRP-immunopositive cells have been colocalized with TH-expressing cells in rat adrenal gland, confirming that PrRP may be produced in the adrenal medulla (Fujiwara *et al.* 2005). Combining these findings suggests that PrRP may play an important role in modulating catecholamine secretion and/or synthesis in a paracrine manner. Indeed, we have shown that PrRP may stimulate catecholamine secretion via the PKA pathway, but not the mitogenic effects in chromaffin cells (Nanmoku *et al.* 2003). However, it is not known whether PrRPs influence the biosynthesis of catecholamine in chromaffin cells.

The signal transduction pathways for the PrRP receptor (GPR10) remain to be determined. Kimura *et al.* (2000) showed that PrRP stimulates the extracellular signal-related kinases (ERKs) in rat pituitary GH3 cells via Gi/Go, and they claimed that this is independent of intracellular Ca²⁺. In addition, activation of the stress-activated protein kinase/c-jun N terminal kinase (SAPK/JNK) by PrRP is dependent on protein kinase C (PKC). Recently, Langmead *et al.* (2000) also demonstrated that

PrRP stimulates the mobilization of intracellular Ca^{2+} from the endoplasmic reticulum in HEK-293-GPR10 cells, supporting the suggestion that the PrRP receptor (GPR10) could be coupled with the Gq-PLC pathway. These results indicate that PrRP may stimulate PKC activity.

In adrenal medullary cells, TH is a rate-limiting enzyme in the biosynthesis of catecholamines. TH activity can be regulated by both short- and long-term mechanisms. Short-term regulation of enzyme activity occurs at the post-translational level. Central to this regulation is the phosphorylation of TH, which results in activation of the enzyme. Indeed, TH is phosphorylated and activated by a variety of protein kinases, including protein kinase C (PKC). Long-term regulation is exerted at the TH protein synthesis level after TH gene transcription (Holz *et al.* 1982, Dunn & Holz 1983, Stoehr *et al.* 1986, Plevin & Boarder 1988).

In the present study, we investigated the effect of PrRP31 and PrRP20 on catecholamine biosynthesis in the rat pheochromocytoma cell line PC12.

Materials and Methods

Reagents

Unless otherwise noted, all reagents were purchased from Wako Seiyaku (Tokyo, Japan). Rat PrRP31 and PrRP20 were obtained from the Peptide Institute (Osaka, Japan) and Phoenix Pharmaceuticals (Mountain View, CA, USA) respectively. The protein kinase C inhibitor, Ro 32-0432 was purchased from Calbiochem (San Diego, CA, USA).

Cells were preincubated for either 30 min with H89 (10 μM) or Ro-32-0432 (100 nM), or 60 min with PD98059 (50 μM) and then exposed to rat PrRP31 (100 nM). The experimental conditions for treatment of the cells with H89, Ro-32-0432 or PD98059 were identical to those described previously (Chabot-Fletcher & Breton 1998, Alessi *et al.* 1995, Marley *et al.* 1995, Hwang *et al.* 1997).

Cell culture

The PC12 cell line (RCB009) was obtained from the RIKEN Cell Bank (Ibaraki, Japan). Cells were grown in 75 cm^2 flasks in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Gaithersburg, MD, USA) containing 10% inactivated horse serum (Gibco BRL) and 10% fetal bovine serum (Gibco BRL) in a humidified atmosphere of 5% CO_2 /95% O_2 at 37 °C. The culture medium was changed three times per week. Cells were removed from the flasks for subculture and for plating into assay dishes, using a Ca^{2+} / Mg^{2+} -free solution containing 172 mM NaCl, 5.4 mM KCl, 1 mM NaH_2PO_4 and 5.6 mM glucose at pH 7.4. After about 2 min in this

solution, the cells were detached by tapping the side of the flask. The cells (1×10^6) were plated onto 35 mm polystyrene dishes and cultured with 2 ml DMEM for 2 days as described above. Cells were then used for experiments in a serum-starved condition (Takekoshi *et al.* 2000).

Tyrosine hydroxylase enzyme activity

Tyrosine hydroxylase enzyme activity was measured by a method previously reported by Kumai *et al.* (1998). Experiments were initiated by replacing the cell culture medium with HEPES-buffered Krebs solution containing various concentrations of test substance and then incubating the cells for 10 min at 37 °C. Cells were homogenized in 0.25 M sucrose (50 volumes) with a glass tissue grinder. The standard incubation medium consisted of the following components in a total volume of 250 μl : 100 μl tissue homogenate, 40 μl of 1 M sodium acetate buffer (pH 6.0), 40 μl of 1 mM L-tyrosine or D-tyrosine, 20 μl of 1 M 6-methyl-5,6,7,8-tetra-hydropterine in 1 M 2-mercaptoethanol, 20 μl of 20 mM catalase and 30 μl water. The medium was incubated at 37 °C for 30 min and then the reaction was stopped with 1 M perchloric acid, containing dihydroxy benzylamine as an internal standard, and 0.2 M EDTA in an ice bath. Then, 1 M potassium carbonate and 0.2 M Tris-HCl (pH 8.5) containing 1% EDTA were added. The 3-(3,4-dihydroxyphenyl)-alanine (DOPA) was extracted by the aluminum oxide method. Forty microliters of extracted medium were mixed with 0.1 N NaOH and TSK-GEL ODS-120T (TOSOH, Japan) and analyzed by HPLC. The mobile phase consisted of the following components: 50 mM sodium acetate, 20 mM citric acid, 12.5 mM sodium octyl sulfate, 1 mM di-*n*-butylamine and 0.134 mM EDTA. All separations were performed isocratically at a flow rate of 0.6 ml/min at 28 °C. The detector potential was maintained at +0.65 V. The TH enzyme activity was calculated as the amount of DOPA formed from tyrosine per milligram protein per minute.

Real-time PCR analysis for TH mRNA level

TH mRNA level was determined by real-time PCR analysis, as described previously by Kitaoka *et al.* (2003). The PCR reaction mixture was prepared with a Taqman PCR master reagent kit (PE Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. The thermal cycling protocol was 2 min at 50 °C and 10 min at 95 °C, and this was followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Thermal cycling, fluorescence detection and data analysis were performed on the ABI PRISM 7700 Sequence Detector with the software provided with the instrument.

The sequences of synthetic oligonucleotide primers and probes were as follows:

- TH, forward primer: AGA GGG ATG GGA ATG CTG TTC
 - reverse primer: CGA GAC AAG GAG GAG GGT TTT
 - TaqMan probe: AAC CTG CTC TTC TCC CTG A.
- For GAPDH detection, we used TaqMan GAPDH Detection Reagents (PE Applied Biosystems).

PKC activity

PKC activity was measured in cultured confluent cells as previously described (Takekoshi *et al.* 2001). Cell cultures (in 90 mm dishes) were incubated in control medium (5% glucose) or with PrRP31 or PrRP20 for 10 min. The cells were then harvested, homogenized by sonification in 20 mM Tris-HCl (pH 7.5), and incubated for 30 min with 100 μ l reaction buffer solution containing a pseudo-substrate and various phospholipids from a commercially available kit (Pep Taq-nonradioactive PKC activity kit; Promega). The reaction was terminated by heating and the reaction mixture was separated into phosphorylated and nonphosphorylated substrates on a 0.9% agarose gel. For quantification, the gel bands were visualized by UV light and then excised, and the absorbance was measured at 570 nm. The results were expressed in OD units

Western blot analysis for ERKs, JNK and p38 MAPK activity

Phosphorylation of ERKs, JNK and p38 MAPK proteins from porcine adrenal medullary cells was measured with an antibody kit (New England Biolabs, Beverly, MA, USA), according to the manufacturer's instructions (Ishii *et al.* 2001). Cells were starved for 4 h and then the medium was replaced with HEPES-buffered Krebs buffer including various test substances. Lysates were immunoprecipitated with anti-ERKs or anti-JNK antibody. Immunoprecipitates were subjected to 7.5% SDS-PAGE and analyzed by immunoblotting with antiphosphotyrosine antibody. We also determined the total amount of ERK, JNK and P38 by using antibodies specific to all forms of ERK, JNK and P38 respectively (pan-ERK). MAPK activity is represented as the relative ratio of the density of phospho-MAPKs to that of total (pan-) ERKs.

Statistical analysis

All data are expressed as means \pm S.E.M. The significance of differences in the data was determined by analysis of variance (ANOVA), and *P* values less than 0.05 were considered significant. The number of replicates indicates the number of separate experiments.

Results

Effect of either PrRP31 or PrRP20 on TH enzyme activity

As shown in Fig. 1, PrRP31 at a concentration of 10 nM, 100 nM or 1 μ M significantly increased TH enzyme

activity by 2.0-, 3.1- and 4.1-fold over the basal value respectively ($P < 0.05$). PrRP20 (100 nM and 1 μ M) also significantly increased TH enzyme activity 1.6- and 1.9-fold over the basal value respectively ($P < 0.05$). The stimulation in TH enzyme activity mediated by PrRP20 was significantly less than that for PrRP31. The PKC inhibitor, Ro-32-0432 (100 nM), significantly inhibited PrRP31-induced TH enzyme activity ($P < 0.05$). Previously, we have demonstrated that the cAMP-PKA system is involved in PrRP31-induced catecholamine release (Nanmoku *et al.* 2003). Thus, we examined the effect of H89 (at 10 μ M), a PKA inhibitor, on PrRP31-induced catecholamine biosynthesis. H89 significantly inhibited PrRP31-induced TH enzyme activity ($P < 0.05$). Neither H89 nor Ro 32-0432 affected the basal TH enzyme activity (data not shown).

Nicotine is known to stimulate catecholamine synthesis in chromaffin cells. Nicotine (10 μ M), which stimulated TH enzyme activity 2.9-fold over the basal value, was used as a convenient positive control in this study. Interestingly, the level of PrRP31 (100 nM)-induced TH enzyme activity was similar to that observed for nicotine.

Effect of either PrRP31 or PrRP20 on TH mRNA expression

As shown in Fig. 2, PrRP31 at a concentration of 10 nM, 100 nM or 1 μ M significantly increased TH mRNA level by 2.3-, 3.6- and 4.0-fold over the basal value respectively ($P < 0.05$). PrRP20 at 10 nM, 100 nM or 1 μ M also increased the TH mRNA level by 1.8-, 2.2- and 3.1-fold over the basal value respectively ($P < 0.05$). However, the PrRP20-stimulated TH-mRNA level was significantly reduced compared with that for PrRP31. Both Ro-32-0432 and H89 inhibited the PrRP31-induced TH mRNA level. Neither H89 nor Ro 32-0432 affected the basal TH mRNA level (data not shown). In PC12 cells, it has been shown that PKC is upstream of Raf, which in turn results in the activation of ERKs (Sozeri *et al.* 1992, Kolch *et al.* 1993). Thus, we examined whether ERKs are involved in PrRP31-induced TH mRNA level. Pretreatment of cells with the MEK-1-inhibitor PD-98059 (50 μ M) significantly reduced the PrRP31 (100 nM)-induced TH mRNA level.

Effect of either PrRP31 or PrRP20 on PKC activity

As shown in Fig. 3, PrRP31 at a concentration of 1 nM, 10 nM, 100 nM or 1 μ M significantly increased PKC activity by 1.4-, 1.7-, 1.9- and 2.3-fold over the basal value respectively ($P < 0.05$). PrRP20 at a concentration of 10 nM, 100 nM or 1 μ M also caused an increase in PKC activity by 1.4-, 1.6- and 1.7-fold over the basal value respectively ($P < 0.05$). However, the PrRP20-stimulated PKC activity was significantly less than that of PrRP31.

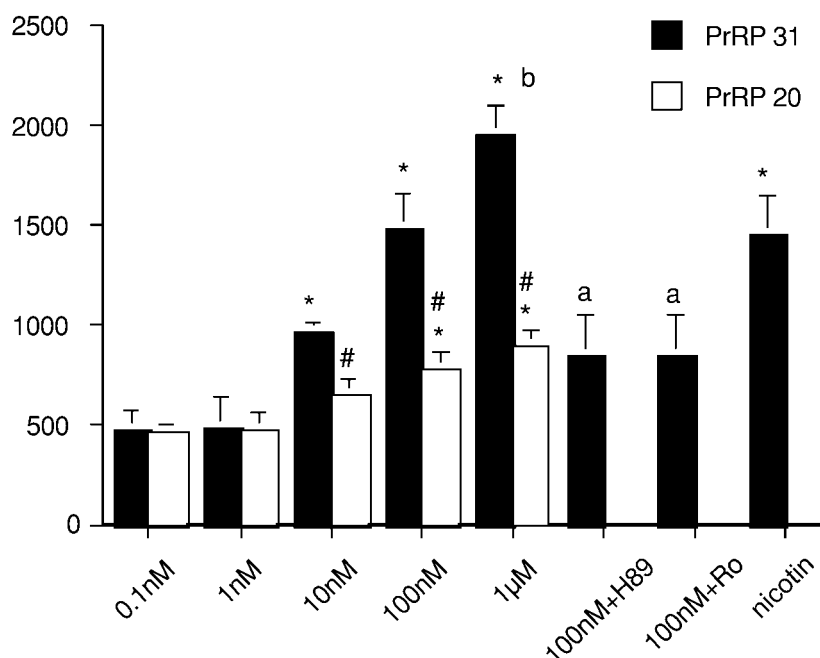


Figure 1 Effect of PrRPs on TH enzyme activity in PC12 cells. Cells were treated with various concentrations (0.1 nM to 1 µM) of rat PrRP20 or PrRP31. Other cells were preincubated with either H89 (10 µM) or Ro-32-0432 (100 nM) for 30 min and then exposed to rat PrRP31 (at 100 nM) for 5 min. In addition, some cells were treated with nicotine (10 µM) in the absence of PrRP. TH enzyme activity was then measured as described in Materials and Methods. The data shown are mean values \pm s.e. ($n=2$) (experiments were carried out twice in triplicate). *Significantly different ($P<0.05$) from the basal value; #significantly different ($P<0.05$) from the value induced by PrRP31; ^asignificantly different ($P<0.05$) from the value induced by PrRP31 (100 nM) alone; ^bsignificantly different ($P<0.05$) from the value induced by nicotine (10 µM) alone.

Effect of PrRP31 on ERKs, JNK and p38 MAPK activity

As shown in Fig. 4, PrRP31 (1 µM) significantly induced increases in ERKs (p44+p42) and JNK (p54+p46) activity by about 2.1- and 2.9-fold respectively. In contrast, PrRP31 did not affect P38 MAPK activity.

Discussion

The results presented in this study show that both PrRP31 and PrRP20 stimulate increased levels of TH enzyme activity and TH mRNA in parallel, suggesting that both mechanisms contribute to PrRP-induced catecholamine biosynthesis (Figs 1 and 2).

We have also demonstrated that both PrRP31 and PrRP20 significantly increase PKC activity in PC12 cells (Fig. 3). Interestingly, the increase in TH enzyme activity and mRNA level evoked by both PrRP31 and PrRP20 was inhibited by 100 nM Ro-32-0432 (Figs 1 and 2). This would suggest that the stimulatory effect of PrRP on TH enzyme activity and mRNA level in chromaffin cells is mediated, at least in part, by the PKC pathway.

It is well established that PKC exerts stimulatory effects on both TH enzyme activity and TH mRNA (Holz *et al.* 1982, Plevin & Boarder 1988). Thus, it is likely that both PrRP31 and PrRP20 stimulate PLC breakdown via its receptor, leading to stimulation of the PKC pathway. This, in turn, results in the stimulation of TH enzyme activity and TH synthesis.

The PKA inhibitor H89 (at 10 µM) attenuated PrRP31-induced TH synthesis (Figs 1 and 2), indicating that the PKA pathway might be involved. These findings agree with our previous report that both PrRP31 and PrRP20 stimulate an increase in the level of cAMP (Takekoshi *et al.* 2003). Taken together, these results suggest that the effects of PrRP are mediated through both the PKA and PKC pathways. This conclusion is supported by the finding that either a PKA inhibitor or a PKC inhibitor moderately, but significantly, inhibit PrRP31-induced TH enzyme activity and TH mRNA accumulation (Fig. 2). Interestingly, the results described here are reminiscent of those for the strong secretagogue pituitary adenylate cyclase-activating polypeptide (PACAP), which is known to be coupled with both the PKA and PKC

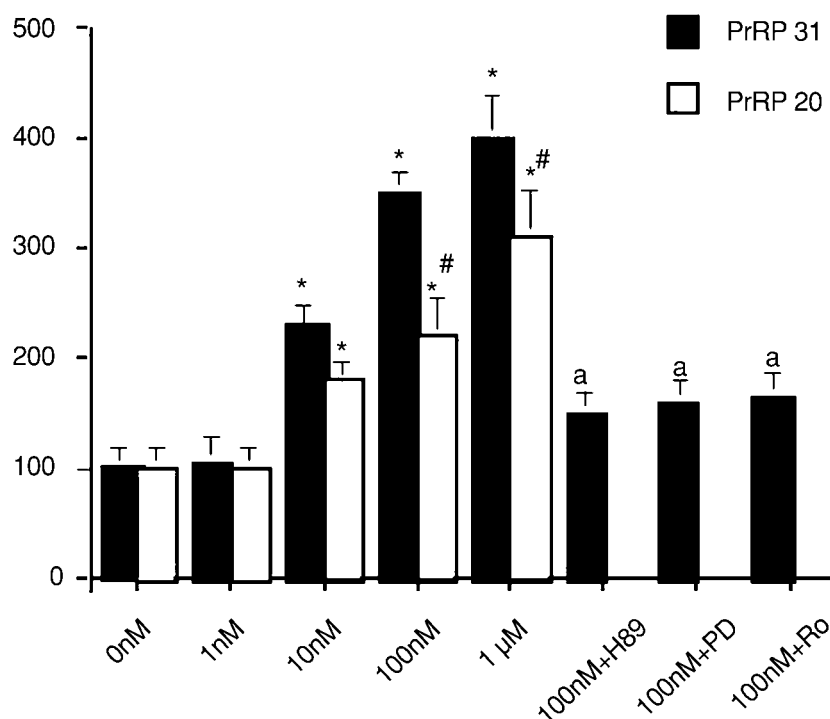


Figure 2 Effect of PrRPs on TH mRNA level in PC12 cells. Cells were treated with various concentrations (0.1 nM to 1 μ M) of rat PrRP20 or PrRP31 for 8 h. Other cells were preincubated for either 30 min with H89 (10 μ M) or Ro-32-0432 (100 nM), or 60 min with PD98059 (50 μ M), and then exposed to rat PrRP31 (100 nM) for 8 h. cDNA from PC12 cells was characterized by a real-time PCR method, as described in Materials and Methods. The data shown are mean values \pm s.e. ($n=2$) (experiments were carried out twice in triplicate). *Significantly different ($P<0.05$) from the basal value; #significantly different ($P<0.05$) from the value induced by PrRP31; ^asignificantly different ($P<0.05$) from the value induced by PrRP31 (100 nM) alone. TH mRNA levels are normalized to GAPDH levels in the same sample.

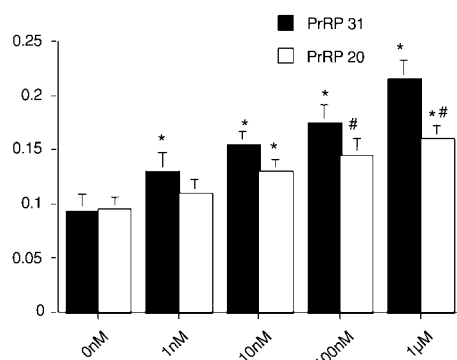


Figure 3 Effect of PrRP on PKC activity in PC12 cells. Cells were incubated with various concentrations (1 nM to 1 μ M) of rat PrRP20 or PrRP31 for 10 min. PKC activity was then measured as described in Materials and Methods. The values represent the means \pm s.e. ($n=2$) (experiments were carried out twice in triplicate). *Significantly different ($P<0.05$) from the basal value; #significantly different ($P<0.05$) from the value induced by PrRP31.

pathways. Indeed, using porcine adrenal medullary cells (Isobe *et al.* 1996), we have previously shown that PACAP stimulates an increase in the level of TH mRNA in both a PKA- and PKC-dependent manner.

We also tested the effect of PrRP31 on a number of key kinase enzymes involved in signal transduction pathways. In our experimental system, PrRP31 stimulated ERK (2.1-fold) and JNK (2.9-fold), but not p38. The underlying mechanism to account for the different results obtained for the three MAPK families remains unclear. Kimura *et al.* (2000) reported that 1 μ M of PrRP31 markedly induced both JNK and ERK activity in rat pituitary GH3 cells. They propose that PrRP activates ERK and JNK, and that both cascades are necessary to elicit prolactin promoter activity. However, they did not examine the effect of PrRP31 on p38 MAP kinase activity.

Mounting evidence suggests that PKC is capable of phosphorylating and activating Raf, which in turn results in the activation of ERKs in PC12 cells. PrRP31 appears to activate JNK in PC12 cells (Sozeri *et al.* 1992, Kolch *et al.* 1993) in a similar fashion to the activation of

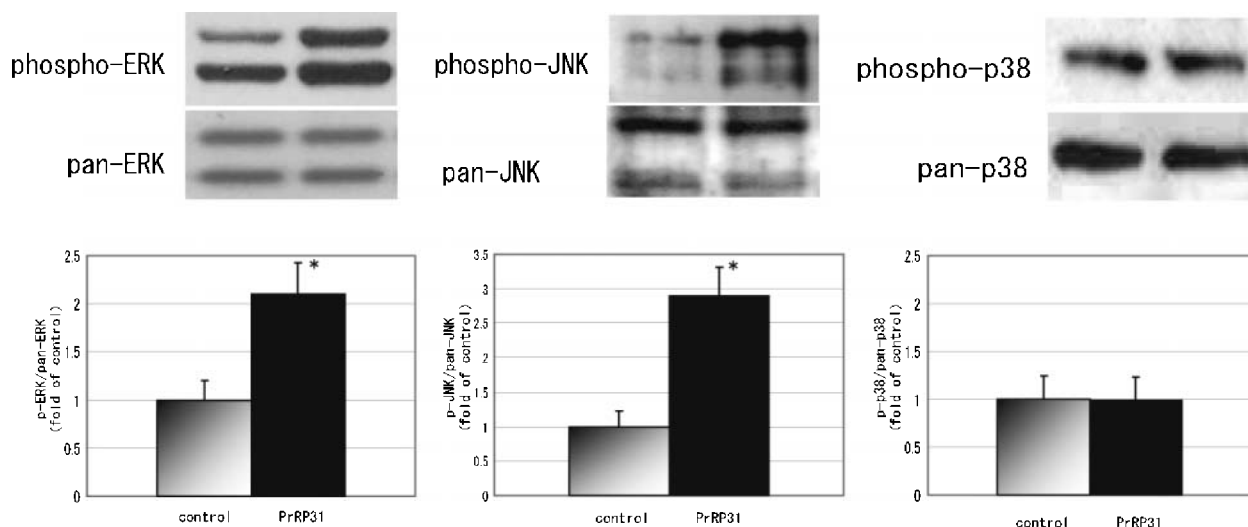


Figure 4 Effect of PrRP31 on ERKs, JNK and p38 MAPK activity. Cells were incubated for 10 min with PrRP31 (1 μ M). ERKs, JNK and p38 MAPK activity was then determined as described in Materials and Methods. Upper panel shows representative data. Lower panel shows summarized data of densitometrical analyses of ERKs activation. MAPK activity is represented as the relative ratio of the density of phospho-MAPKs against that of total (pan-) ERKs. Relative ratio in control was expressed as 1 arbitrary unit. $n=2$; experiments were carried out twice in duplicate. * $P<0.05$.

ERKs. Indeed, *c-fos* protein can be phosphorylated at Ser 374 by ERK (Chen *et al.* 1993). In addition, other kinases, including Fos-regulating kinase (FRK), PKA and ribosomal S-6 kinase, have been shown to phosphorylate *c-fos* protein (Deng & Karin 1994, Taylor *et al.* 1993). JNK phosphorylates *c-jun* at Ser 63 and Ser 73 (Hibi *et al.* 1993). Transcriptional activation of TRE (tetradecanoyl phorbol 13-acetate (TPA)-responsive elements), containing promoters of genes such as TH, is mediated by the AP-1 complex, composed of different Fos/Jun families (Le Bourdelles *et al.* 1988, Gizang-Ginsberg & Ziff 1994, Whitmarsh & Davis 2000). Thus, the activation of ERKs induced by PrRP appears to be important in regulating TH transcription through AP-1 formation. Our observation that the PrRP31-induced increase in TH-mRNA is significantly inhibited by PD-98059 supports this proposal.

Although the physiologic role of the peptide other than its prolactin-releasing properties remains unclear (Matsumoto *et al.* 1999), Samson *et al.* (2000) have shown that intracerebroventricular administration of both PrRP31 and PrRP 20 significantly increases the blood pressure in a conscious rat. Apart from this 'central effect' of PrRP, we have shown that PrRP directly stimulates both catecholamine synthesis and secretion in the adrenal medulla. Thus, it is tempting to speculate that the 'peripheral actions' of PrRP on the adrenal medulla could be responsible, at least in part, for the stimulatory effect of PrRP on blood pressure under physiologic conditions. Further studies, involving the *in vivo* administration of PrRP, will be needed to clarify to what extent the 'peripheral actions' of PrRP contribute to regulating blood pressure via catecholamine secretion from the adrenal medulla.

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