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Activation of mitogen-activated protein kinase by the nociceptin receptor expressed in Chinese hamster ovary cells

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Abstract Activation of the nociceptin receptor stably expressed in Chinese hamster ovary cells induced a transient mitogen-activated protein kinase (MAPK) activation, via pertussis toxin-sensitive G-proteins. The nociceptin receptor-mediated MAPK activation was partially blocked by down-regulation or inhibition of protein kinase C, and suppressed by pretreatment with a phosphatidylcholine-specific phospholipase C inhibitor, D609. Furthermore, a tyrosine protein kinase inhibitor, genistein, and phosphatidylinositol 3-kinase inhibitors, wortmannin and LY294002, affected the nociceptin-induced MAPK activity. The nociceptin-induced MAPK activation may lead to activation of phospholipase A_2 and induce changes in gene expression.

Key words: Nociceptin receptor; cDNA expression; Mitogen-activated protein kinase; Chinese hamster ovary cell

1. Introduction

The opioid receptor mediates a variety of pharmacological effects of opioid analgesics. cDNAs encoding the three major types of the opioid receptor, δ, μ and κ, have been cloned and sequenced [1]. In addition, several laboratories have cloned the cDNA encoding an opioid receptor homolog, ORL1 [2], ROR-C [3] or LC132 [4], by cross-hybridization or polymerase chain reaction. Recently, a peptide nociceptin [5], also designated as orphanin FQ [6], has been isolated from brain tissue, on the basis of its ability to inhibit adenylate cyclase in Chinese hamster ovary (CHO) cells stably transfected with the ORL1 cDNA. Nociceptin is constituted from 17 amino acid residues and its amino acid sequence resembles that of an endogenous opioid peptide, dynorphin A [5,6]. When injected intracerebroventricularly or intrathecally into mice, nociceptin induced hyperalgesia and allodynia, in contrast to antinociceptive action of opioid peptides [5–7]. Thus, despite its structural similarity to opioid peptides, nociceptin appears to be pharmacologically and physiologically distinct from them.

Molecular basis for the nociceptin actions has been examined using neuronal cells endogenously expressing the nociceptin receptor. Electrophysiological studies demonstrated that activation of the nociceptin receptor expressed in hippocampal neurons and a neuroblastoma cell line leads to inhibition of the voltage-dependent Ca^{2+} channel activity [8,9]. Furthermore, it has been shown that nociceptin increases inwardly rectifying K^+ conductance in dorsal raphe nucleus neurons [10].

Mitogen-activated protein kinase (MAPK), also known as extracellular signal-regulated kinase (ERK) is a central component of an evolutionarily conserved kinase cascade, which integrates various extracellular signals converging to it [11,12]. It has been shown that MAPK is activated by G-protein-coupled receptors as well as tyrosine kinase receptors [12]. We have previously reported that activation of the δ-, μ- and κ-opioid receptors expressed in CHO cells elicits MAPK activation through the action of pertussis toxin (PTX)-sensitive G-proteins [13]. In this investigation, in order to gain a further insight into the molecular basis of the nociceptin action, we examined functional coupling of the nociceptin receptor with MAPK. The results obtained indicate that activation of the nociceptin receptor expressed in CHO cells induces MAPK activation via PTX-sensitive G-proteins.

2. Materials and methods

2.1. cDNA expression

The 1.7-kb *Hin*dIII fragment from the plasmid pROR30 [3], carrying the entire protein coding sequence of the nociceptin receptor (ROR-C), was cloned into an expression vector pcDNA3 (Invitrogen) in the same orientation with respect to the cytomegalovirus promoter to generate pcDNA3ROR-C.

CHO cells were transfected with pcDNA3ROR-C by the calcium phosphate method [14]. To isolate clones expressing the nociceptin receptor, clones resistant to 600 μg/ml G418 were screened by RNA blot hybridization analysis.

2.2. [^{3}H]Nociceptin binding assay

Crude membranes were prepared as in [13]. Binding reaction was performed with membrane preparations (15-30 μg of protein) in 0.2 ml of 50 mM Tris-HCl, pH 7.5, containing 0.1% bovine serum albumin at 30°C for 1 h in the presence of various concentrations of [^{3}H]nociceptin (173 Ci/mmol; Amersham). After incubation, the samples were collected on polyethyleneimine-treated GF/B filters (Whatman) and washed with 10 ml of ice-cold 50 mM Tris-HCl, pH 7.5. The filters were then counted for radioactivity. Non-specific binding was determined with 1 μM nociceptin. The protein concentration in the membrane preparation was measured as in [15].

2.3. Assay of MAPK activity

Cells were grown to subconfluence in 60-mm dishes, serum-starved for 24 h, and stimulated by nociceptin at 37°C for the indicated times. Preparation of cell lysates and assay for the MAPK activity were performed as in [13]. The protein concentration in the cell lysate was measured as in [15], and the MAPK activity was expressed as pmol/min/mg protein.

2.4. Immunoblot analysis

Cell lysates were prepared as in [13]. Samples were electrophoresed on an SDS-10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After blocking for 1 h in the buffer TBS-T

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Abbreviations: CHO, Chinese hamster ovary; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PTX, pertussis toxin; MEK, MAPK/ERK kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PC-PLC, phosphatidylcholine-specific phospholipase C; PC, phosphatidylcholine; PI3K, phosphatidylinositol 3-kinase

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(20 mM Tris-HCl (pH 7.5), 137 mM NaCl and 0.1% Tween-20) containing 5% non-fat dry milk, the membrane was incubated for 2 h at room temperature with phospho-specific MAPK antibody (New England Biolabs) diluted 1:1000 in TBS-T containing 5% non-fat dry milk, and then incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody. After washing with TBS-T, the protein–antibody complex was visualized using the enhanced chemiluminescence detection system (Amersham).

2.5. Statistics

Data are expressed as mean ± SEM. Statistical analyses of data were performed by ANOVA followed by Fisher's protected least significance difference test. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

CHO cells were stably transfected with the cloned cDNA for the nociceptin receptor (ROR-C) [3], and a clone, CROR-C39, was selected for further analysis. The membrane preparation from CROR-C39 was subjected to the [^{3}H]nociceptin binding assay. Fig. 1 shows that ROR-C expressed in CHO cells bound nociceptin with a high affinity, whereas negligible binding was found for non-transfected CHO cells. Scatchard analysis demonstrated that the K_d value for nociceptin of the expressed receptor is 1.2 ± 0.1 nM (mean ± SEM; $n = 3$), and that the B_{max} value is 5.1 ± 0.8 pmol/mg protein ($n = 3$).

As shown in Fig. 2A, MAPK activity in cell lysates prepared from CROR-C39 was increased to ~10-fold of the non-stimulation control level at 3-5 min after the addition of 100 nM nociceptin, and thereafter declined gradually. On the other hand, 100 nM nociceptin did not elicit significant MAPK activation in non-transfected CHO cells (data not shown). The MAPK activity evoked by stimulation with nociceptin for 5 min was dose-dependent (Fig. 2B), and the maximal activation (10.4 ± 2.2-fold of the non-stimulation control value, $n = 4$) was observed at 1 μM nociceptin. The EC_{50} value was 11.1 ± 1.7 nM ($n = 4$). Treatment of the cells with 100 ng/ml PTX for 12 h completely abolished the ability of nociceptin to activate MAPK (Fig. 2C), indicating that the response is mediated by PTX-sensitive G-proteins (G_i and/or G_o). An opioid antagonist naloxone (10 μM) did not significantly affect the nociceptin-induced MAPK activity (Fig. 2C), in accordance with the reports that the nociceptin receptor does not show high affinity for opioid ligands, in spite of high homology in amino acid sequence between the nociceptin receptor and the opioid receptors [2–4].

MAPKs, consisting of two isoforms p44 (ERK1) and p42 (ERK2), exist as unphosphorylated forms in non-stimulated cells and become activated when both tyrosine and threonine residues are phosphorylated by MAPK/ERK kinase (MEK) [11,12,16]. Consistently, pre-incubation of the cells with 50 μM PD98059 [17], a specific MEK inhibitor, for 2 h almost completely abolished the nociceptin-induced MAPK activity (Fig. 2C). Furthermore, as shown in Fig. 3A, stimulation of CROR-C39 cells with nociceptin induced appearance of phosphorylated forms of p44 and p42 MAPKs in a time-dependent manner, whereas phosphorylated MAPKs were not detectable in non-stimulated control cells. p44 and p42 MAPKs became maximally phosphorylated at 3-5 min of stimulation. Fig. 3B shows that the appearance of phosphorylated MAPKs is dependent on the nociceptin concentration. Maximal phosphorylation of p44 and p42 MAPKs was observed in the range from 100 nM to 1 μM nociceptin. Thus, the time course and

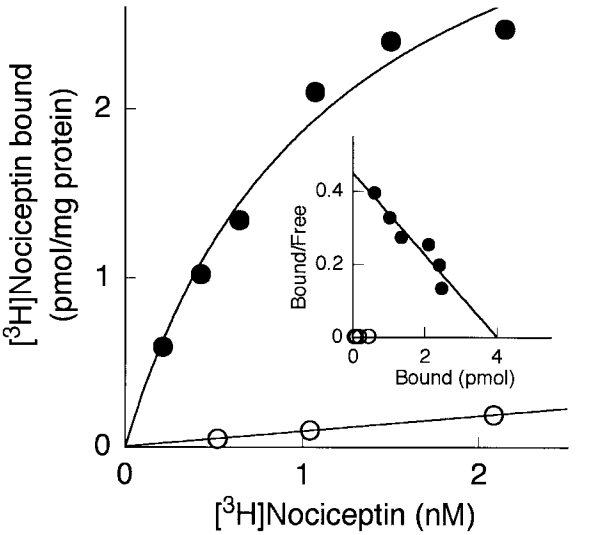

Fig. 1. Saturation analysis of [^{3}H]nociceptin binding. Membrane preparations from CHO cells expressing the nociceptin receptor, CROR-C39 (●), and non-transfected CHO cells (○) were subjected to saturation analysis of [^{3}H]nociceptin binding. The inset shows Scatchard plot of the data. Representative result from three separate experiments is shown.

the dose dependence of nociceptin-induced MAPK phosphorylation paralleled with those of the nociceptin-induced increase in MAPK activity.

As shown in Fig. 4A, the MAPK activation induced by nociceptin was partially (~25%) suppressed by protein kinase C (PKC) down-regulation induced by treatment with 1 μM phorbol 12-myristate 13-acetate (PMA) for 18 h. Fig. 4A also shows that PKC inhibition by pretreatment for 4 h with 1 μM GF 109203X [18], partially (~25%) inhibited the nociceptin-induced MAPK activation. These results suggest that the MAPK activation mediated by the nociceptin receptor is at least partially dependent on PKC activation. It is not likely that the nociceptin receptor induces PKC activation through production of diacylglycerol from phosphatidylinositol 4,5-bisphosphate, because nociceptin did not induce significant elevation of the cellular level of inositol 1,4,5-trisphosphate (data not shown), indicating that phosphatidylinositol-specific phospholipase C is not significantly activated by nociceptin. Therefore, we tested the possible involvement of phosphatidylcholine-specific phospholipase C (PC-PLC), since it has been reported that diacylglycerol derived from phosphatidylcholine (PC) can activate PKC [19]. Pretreatment of the cells for 2 h with 50 μM D609 [20], a specific PC-PLC inhibitor, suppressed the nociceptin-induced MAPK activation (Fig. 4A). This result suggests the involvement of PC-PLC in the signaling pathway of MAPK activation mediated by the nociceptin receptor.

Pretreatment of CROR-C39 cells for 2 h with 200 μM genistein, a tyrosine protein kinase inhibitor, partially inhibited the nociceptin-induced MAPK activation, although statistically not significant (Fig. 4A). In the cells in which PKC was down-regulated by treatment with 1 μM PMA for 18 h, genistein reduced the nociceptin-induced MAPK activity nearly to the prestimulation level, suggesting that tyrosine protein kinase is involved in the PKC-independent component of the nociceptin-evoked MAPK activation. Fig. 4B demonstrates that the nociceptin-induced MAPK activation was par-

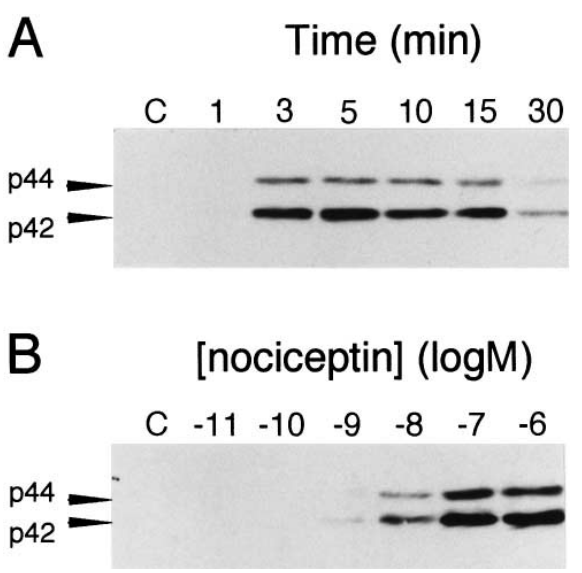

Fig. 3. Immunoblot analysis using the antibody specific for the phosphorylated MAPKs. CHO cells expressing the nociceptin receptor, CROR-C39, were stimulated for the indicated times with 100 nM nociceptin (A), and for 5 min with the indicated concentrations of nociceptin (B). Cell lysates were subjected to immunoblot analysis using the phospho-specific MAPK antibody. Representative results from three separate experiments are shown. The positions of phosphorylated forms of MAPKs (p44 and p42) are as indicated. *C*, non-stimulation control.

tially (30-40%) inhibited by pretreatment of the cells for 2 h with phosphatidylinositol 3-kinase (PI3K) inhibitors, 1 μM wortmannin [21] or 1 μM LY294002 [22]. In the cells pretreated with 1 μM PMA for 18 h to down-regulate PKC, wortmannin almost completely suppressed the nociceptin-induced MAPK activation. These results suggest that PI3K takes part in the nociceptin-induced MAPK activation through the PKC-independent pathway.

4. Discussion

G-protein-coupled receptors mediate activation of MAPKs via the heterotrimeric G-protein [12] through different mechanisms. MAPK activation by the G_i-coupled receptors, including the m2 muscarinic acetylcholine receptor and the α_{2A} adrenergic receptor, is mediated by the βγ subunits of the G-proteins, Ras and Raf kinase in a PKC-independent manner, and is inhibited by pretreatment with tyrosine protein kinase inhibitors [23,24]. On the other hand, it has been recently shown that the m1 muscarinic acetylcholine receptor and the platelet-activating factor receptor couple with G_o to activate MAPK by a PKC-dependent and Ras-independent mechanism [25]. Therefore, our finding that the nociceptin receptor mediates MAPK activation through the action of PTX-sensitive G-proteins (G_i and/or G_o) via PKC-dependent and -independent pathways may suggest that the nociceptin receptor couples with G_o and G_i, which activates MAPK through PKC-dependent and -independent mechanism, respectively. Moreover, the involvement of tyrosine protein kinase and PI3K in the PKC-independent pathway of the nociceptin-induced MAPK activation agrees with the recent report

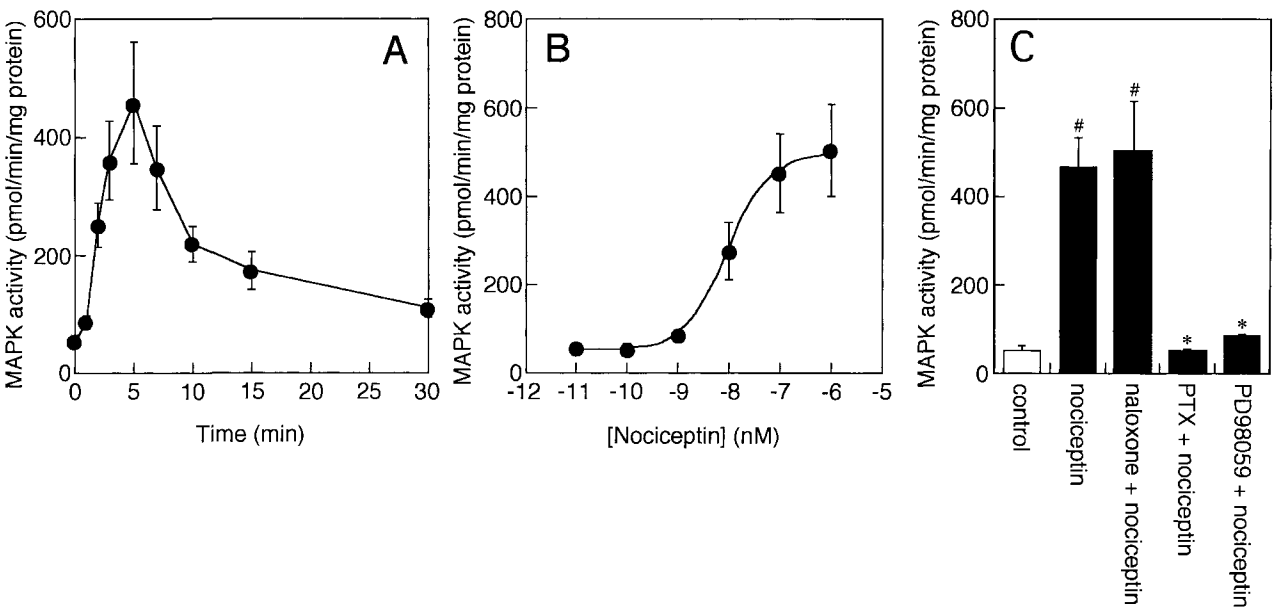

Fig. 2. MAPK activity induced by activation of the nociceptin receptor expressed in CHO cells. A: Time course of the nociceptin-induced MAPK activity. CHO cells expressing the nociceptin receptor, CROR-C39, were stimulated by 100 nM nociceptin for the indicated times. Each point represents the mean ± SEM from four separate experiments. B: Dose-response relationship for the nociceptin receptor-mediated MAPK activation. CROR-C39 cells were stimulated by nociceptin at the indicated concentrations for 5 min. Each point represents the mean ± SEM from eight separate experiments. C: Effects of naloxone, PTX and PD98059 on the nociceptin-induced MAPK activation. CROR-C39 cells were treated with 100 ng/ml PTX for 12 h, 10 μM naloxone for 10 min or 50 μM PD98059 for 2 h, and then stimulated with 100 nM nociceptin for 5 min. Data are mean ± SEM values from eight separate experiments. Significantly different from the control value with neither pretreatment nor nociceptin stimulation; *significantly different from the MAPK activity evoked by nociceptin without pretreatment.

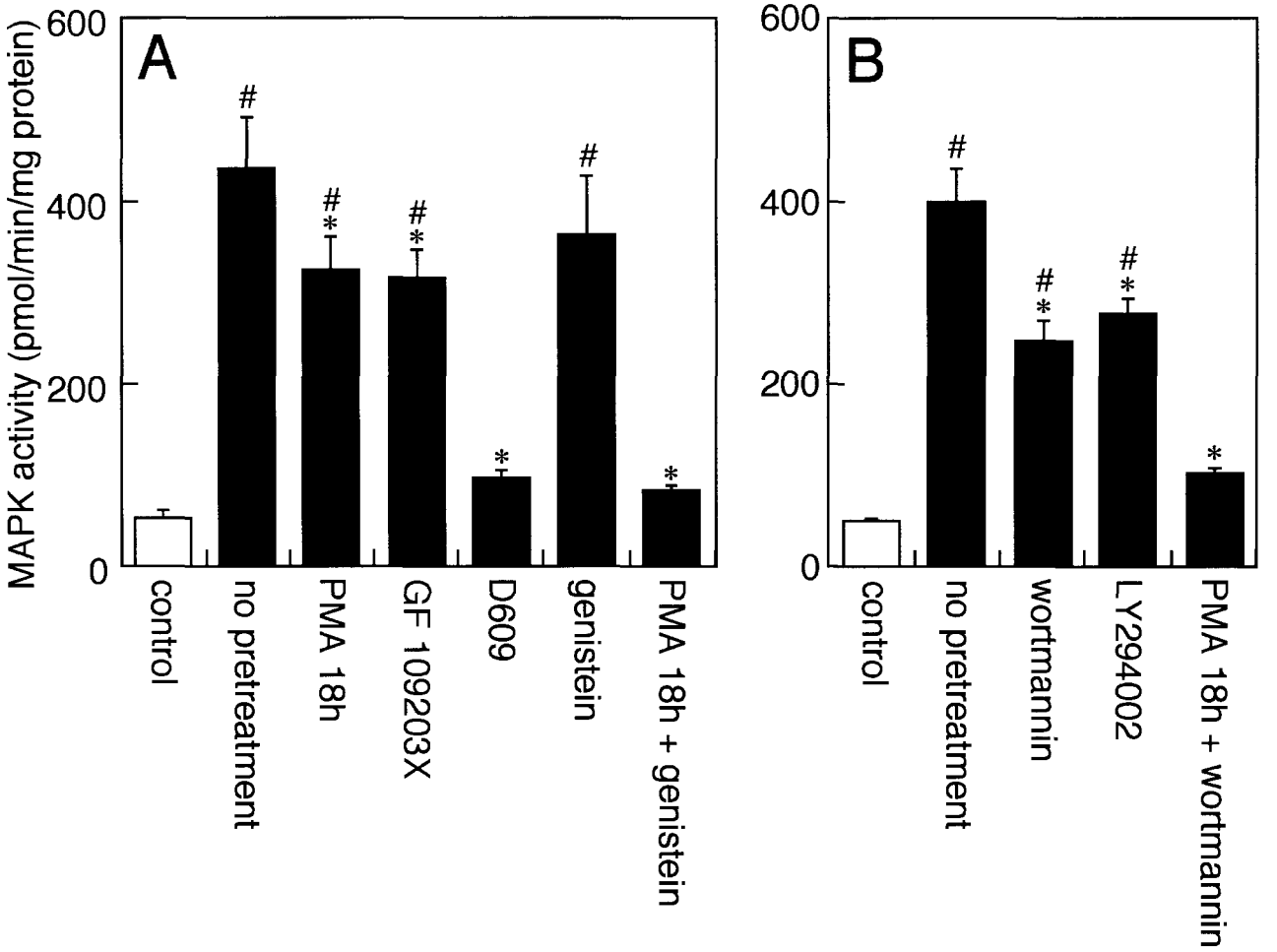

Fig. 4. Effects of pretreatments on the nociceptin-induced MAPK activity. With or without pretreatment, CHO cells expressing the nociceptin receptor, CROR-C39, were stimulated for 5 min with 100 nM nociceptin. Data are mean ± SEM values from eight separate experiments. #Significantly different from the control value with neither pretreatment nor stimulation; *significantly different from the MAPK activity evoked by nociceptin without pretreatment. A: Effects of inhibition of PKC, PC-PLC, and tyrosine protein kinase on the nociceptin-induced MAPK activity. Pretreatment of the cells were as follows: 1 μM PMA for 18 h; 1 μM GF 109203X for 4 h; 50 μM D609 for 2 h; 200 μM genistein for 2 h; the combination of 1 μM PMA for 18 h and 200 μM genistein for 2 h. B: Effects of PI3K inhibitors on the nociceptin-induced MAPK activity. Pretreatment of the cells were as follows: 1 μM wortmannin for 1 h; 1 μM LY294002 for 1 h; the combination of 1 μM PMA for 18 h and 1 μM wortmannin for 1 h.

that PI3Kγ mediates MAPK signaling activated by G-protein βγ subunits in a tyrosine protein kinase-dependent fashion [26].

D609, an inhibitor of PC-PLC [20], suppressed the MAPK activity induced by nociceptin, suggesting that PKC is activated by diacylglycerol derived from PC, resulting in PKC-dependent MAPK activation. This finding agrees with the recent report that PC-PLC represents a component of the mechanism by which MAPK is activated by the 5-hydroxytryptamine$_{1A}$ receptor expressed in CHO cells [27]. The inhibitory effect of D609 on nociceptin-induced MAPK activity was more remarkable than that of PKC inhibition by GF 109203X and PKC down-regulation by prolonged treatment with PMA. This result may suggest that PC-PLC is also involved in the PKC-independent pathway. In agreement with this speculation, it has been reported that PC hydrolysis by PC-PLC acts upstream of Raf kinase and serves to couple Ras to Raf kinase activation, suggesting the possibility that PC-derived diacylglycerol functions directly as a Raf kinase activator without PKC activation [28]. Further study is required to elucidate the mechanism for PC-PLC activation mediated by the nociceptin receptor.

Activation of MAPKs by the nociceptin receptor in neuronal cells may lead to important cellular responses. Cytosolic phospholipase A_2, which hydrolyzes arachidonic acid from membrane phospholipids, represents an important target of the MAPK signal transduction pathway, and activated by phosphorylation by MAPKs [16]. Therefore, activation of the nociceptin receptor may induce an increase in production of eicosanoids, by activation of phospholipase A_2 via MAPKs. Moreover, MAPKs phosphorylate and regulate a variety of transcription factors, including c-Myc, c-Jun, c-Fos and ATF-2 [16]. Thus, it is conceivable that activation of the nociceptin receptor induces alterations in gene expression via MAPK activation, resulting in long-term changes in neuronal function.

In conclusion, our results indicate that the nociceptin receptor expressed in CHO cells functionally couples with the MAPK cascade in a PTX-sensitive manner, and suggest that

PKC, PC-PLC, tyrosine kinase and PI3K are involved in the nociceptin-induced MAPK activation.

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