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Phorbol ester blocks the increase of a high affinity GTPase activity induced by δ_2-opioid receptor agonist in the mouse spinal cord

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Abstract

The high affinity GTPase activity in the mouse spinal cord was increased in a concentration-dependent manner by a selective δ_2-opioid receptor agonist, [D-Ala^2]deltorphin II (0.1–1 μM). This increase of GTPase activity induced by [D-Ala^2]deltorphin II was completely blocked by co-incubation with a selective δ_2-opioid receptor antagonist, naltriben (0.1 μM). A protein kinase C activator, phorbol 12,13-dibutyrate (PDB; 0.1–10 μM), which given alone had no effect on basal GTPase activity, blocked dose-dependently the increase of GTPase activity induced by [D-Ala^2]deltorphin II (1 μM). Our results indicate the possibility that activation of protein kinase C by phorbol ester uncouples the δ_2-opioid receptor from G-proteins in the spinal cord.

Keywords: δ-Opioid receptor; GTPase; Protein kinase C

Desensitization is defined as a decrease in receptor responsiveness. For guanine nucleotide binding proteins (G-protein)-coupled receptors, it has been proposed that desensitization is mediated by the uncoupling of the receptors from G-proteins which is characterized by the activation of protein kinases and phosphorylation of the receptor proteins, leading to impaired activation of G-proteins (Law, 1995). The δ_2-opioid receptor which has been cloned and well characterized by pharmacological studies (Evans et al., 1992; Reisine, 1995; Porreca et al., 1995) is a member of the G-protein-coupled receptor superfamily. We found in the previous study that intrathecal (i.t.) pretreatment of mice with a protein kinase C activator, phorbol 12,13-dibutyrate (PDB), attenuated the antinociception induced by i.t.-administered δ_2-opioid receptor agonist [D-Ala^2]deltorphin II (Narita et al., 1996). These findings suggest that the activation of protein kinase C may phosporylate the δ_2-opioid receptors, resulting in the desensitization of the δ_2-opioid receptor-mediated responses in the mouse spinal cord.

Receptors that interact with G-proteins produce an increase in GTP hydrolysis, ultimately via an increase in the GTPase activity of Gα, but initially by stimulating the binding of GTP to G_α (Law, 1995). Thus, a study of the ability of various receptor agonists to stimulate GTPase activity is useful to determine the nature of the interaction between the G-protein and receptor. The present study was therefore designed to study the effect of PDB on the stimulation of high affinity GTPase activity induced by [D-Ala^2]deltorphin II in the mouse spinal cord. We report here for the first time that activation of protein kinase C by PDBu uncouples the δ_2-opioid receptor from G-proteins in the mouse spinal cord.

Male ICR mice weighing 25–30 g (Sasco, Omaha, NE, USA) were used. Animals were killed by decapitation and their spinal cord was quickly excised on an ice-cold Petri dish. The spinal cord was homogenized in 15 vols. (w/v) of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EGTA, 1 mM dithiothreitol and 1 μM okadaic acid with a Potter-Elvehjem tissue grinder. The homogenates were centrifuged at 4°C for 10 min at $1000 \times g$. The pellets were discarded and the supernatants were centrifuged at 4°C for 20 min at $20000 \times g$. The resuspended pellet was then stored at −70°C until the GTPase assay. The reaction mixture of each GTPase assay consisted of

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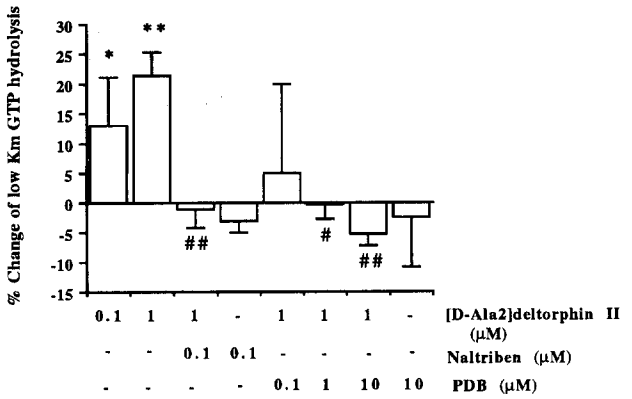

Fig. 1. Inhibition of [D-Ala^2]deltorphin II-stimulated high affinity GTPase activity by naltriben and PDB in spinal cord membranes of the mouse. The suspensions of the spinal membrane were incubated with [^{32}P]GTP in the presence or absence of drugs. Data were expressed as mean of % change of low K_m GTP hydrolysis with S.E.M. from 3–5 independent experiments. The basal GTPase activity was 28.8 ± 2.0 pmol/mg protein/min. ** $P < 0.01$, compared with control. $^{\#,\#\#}$ $P < 0.05$, 0.01, compared with 1 μM [D-Ala^2]deltorphin II.

100000 cpm of [γ-^{32}P]GTP (6000 Ci/mmol; New England Nuclear, Boston, MA, USA), 1 or 50 μM GTP, 0.5 mM adenosine-5′-β-γ-imidotriphosphate (AppNHp), 1 mM ATP, 1 mM oubain, 5 mM creatine phosphate, 2.5 U creatine phosphokinase, 0.01% bovine serum albumin, 120 mM NaCl, 0.2 μM okadaic acid, 0.2 mM dithiothreitol, 0.4 mM EGTA and 50 mM Tris-HCl buffer (pH 7.4) prepared on ice. Naltriben (a gift of Dr. Hiroshi Nagase, Toray, Japan), PDB (Calbiochem-Novabiochem International, San Diego, CA, USA) or [D-Ala^2]deltorphin II (Molecula Research Laboratories, Durham, NC, USA) was added in the assay mixture. The reaction was started by the addition of the membrane (8 μg) to the reaction mixture and incubated 20 min at 30°C. The reaction was terminated by the addition of 5% (w/v) activated charcoal in 20 mM H_3PO_4 and then the ^{32}P released by GTP hydrolysis in supernatants was counted after microcentrifugation. The high affinity (low K_m) GTPase activities were calculated by subtracting the activities with 50 μM GTP (low affinity GTPase) from that with 1 μM GTP (total GTPase). All assays were carried out in triplicate. The data are expressed as means and S.E.M. The statistical significance of differences between groups was assessed with Newman-Keuls multiple comparison test.

As shown in Fig. 1, high affinity (low K_m) GTPase activities in the spinal cord were increased in a concentration-dependent manner by [D-Ala^2]deltorphin II. However, low affinity GTPase activities were not significantly changed by [D-Ala^2]deltorphin II (data not shown). [D-Ala^2]deltorphin II at 0.1 and 1 μM significantly increased the low K_m GTP hydrolysis by 13.0 ± 8.2 and 21.9 ± 3.9% ($P < 0.05$ and < 0.01), respectively, above the control. The increase of GTPase activity induced by [D-Ala^2]deltorphin II (1 μM) was completely inhibited by co-incubation with naltriben (0.1 μM). Naltriben (0.1 μM) added alone did not have any effect on the basal low K_m GTP hydrolysis. PDB at concentrations from 0.1 to 10 μM dose-dependently attenuated the increase of high affinity of GTPase activity induced by [D-Ala^2]deltorphin II (1 μM). PDB even at 10 μM added alone did not affect the basal low K_m GTP hydrolysis.

In the present study, we clearly demonstrate that [D-Ala^2]deltorphin II causes an increase of high affinity GTPase activity in the spinal cord. The effect was mediated by the stimulation of δ_2-opioid receptors because the effect was blocked by δ_2-opioid receptor blocker, naltriben. The increase of high affinity GTPase activity by [D-Ala^2]deltorphin II may result from the activation of G-proteins which coupled to δ_2-opioid receptors.

It has been proposed that GTPγS, a non-hydrolyzable GTP analog, displaces GDP which binds to G_α. The G_α-GTPγS complex is then irreversibly uncoupled from cell surface receptors which results in a decrease of binding ability of ligand to receptors (Law, 1995). We found in the previous studies using synaptic membranes of the mouse spinal cord that the treatment with GTPγS attenuated the specific bindings of [^{3}H][D-Ser^2,Leu^5]enkephalin-Thr^6 (DSLET), a selective δ_2-opioid receptor ligand (Narita et al., 1995). Additionally, i.t. pretreatment with pertussis toxin for 96 h dose-dependently attenuated the antinociception induced by i.t.-administered [D-Ala^2]deltorphin II (Mizoguchi et al., 1996). Taken together, these findings provide strong evidence that high affinity δ_2-selective binding sites in the mouse spinal cord are functionally coupled to Gi/Go proteins.

We found in the present study that the activation of protein kinase C by PDB, which given alone had no effect on the GTPase activity, blocked the increase of high affinity GTPase activity induced by δ_2-opioid receptor agonist in the mouse spinal cord. Based on cloning studies, a number of potential phosphorylation sites by protein kinases are present in cloned δ-opioid receptors (Evans et al., 1992). Indeed, phorbol esters stimulate phosphorylation of the δ-opioid receptor in human embryonic kidney 293 cells (Pei et al., 1995). Our present results, therefore give support to the possibility that phosphorylation of δ_2-opioid receptors induced by the activation of protein kinase C is the mechanism for the uncoupling of the δ-opioid receptor from G-proteins, leading to a decrease in receptor responsiveness in the mouse spinal cord. This contention is supported by our previous findings that i.t. pretreatment of mice with PDBu attenuated the antinociception induced by i.t.-administered [D-Ala^2]deltorphin II and co-incubation of the spinal synaptic membrane with PDB reduced δ_2-opioid receptor bindings (Narita et al., 1996).

It is concluded that the increase of GTPase activity induced by [D-Ala^2]deltorphin II is mediated by the stimulation of δ_2-opioid receptors in the mouse spinal cord and the increase of GTPase activity is blocked by protein kinase C activator, phorbol esters.

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