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Role of protein kinase C in desensitization of spinal δ-opioid-mediated antinociception in the mouse

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1 Receptor phosphorylation and down-regulation by protein kinases may be a key event initiating desensitization. The present studies were designed to investigate the effect of a potent protein kinase C (PKC) activator, phorbol 12,13-dibutyrate (PDBu), on antinociception induced by intrathecal (i.t.) administration of a selective δ-opioid receptor agonist [D-Ala^2]deltorphin II in the male ICR mouse and on the specific binding of [^{3}H]-[D-Ser^2, Leu^5]enkephalin-Thr^6 (DSLET), a δ-opioid receptor ligand, in the crude synaptic membrane of the spinal cord.

2 Intrathecal (i.t.) pretreatment with PDBu at low doses, which injected alone did not affect the basal tail-flick latency, dose-dependently attenuated the antinociception induced by i.t. administration of [D-Ala^2]deltorphin II. The attenuation of i.t.-administered [D-Ala^2] deltorphin II-induced antinociception by PDBu was reversed in a dose-dependent manner by i.t. concomitant pretreatment with a specific PKC inhibitor, calphostin C.

3 In the binding experiment, incubation of the crude synaptic membrane of the spinal cord for 2 h at 25°C with PDBu (0.03 to 10 μM) caused a dose-dependent inhibition of the [^{3}H]-DSLET binding. Scatchard analysis of [^{3}H]-DSLET binding revealed that PDBu at 10 μM displayed a 30.7% reduction in the number of [^{3}H]-DSLET binding sites with no significant change in affinity, compared with the non-treatment control, indicating that the activation of membrane-bound PKC by PDBu causes a decrease in the number of specific δ-opioid agonist binding sites.

4 An i.t. injection of [D-Ala^2]deltorphin II produced an acute antinociceptive tolerance to the antinociceptive effect of a subsequent i.t. challenge of [D-Ala^2]deltorphin II. Concomitant pretreatment with calphostin C markedly prevented the development of acute tolerance to the i.t.-administered [D-Ala^2]deltorphin II-induced antinociception. On the other hand, a highly selective protein kinase A (PKA) inhibitor, KT5720, did not have any effect on the development of acute tolerance to [D-Ala^2]deltorphin II antinociception.

5 These findings suggest that a loss of specific δ-agonist binding by the activation of PKC by PDBu is involved in the PDBu-induced antinociceptive unresponsiveness to δ-opioid receptor agonist in the mouse spinal cord. Based on the acute tolerance studies, we propose that PKC, but not PKA, plays an important role in the process of homologous desensitization of the spinal δ-opioid receptor-mediated antinociception.

Keywords: Phorbol ester; protein kinase C; δ-opioid receptor; antinociception; tolerance; desensitization; phosphorylation

Introduction

A δ-opioid receptor has been cloned (Evans *et al.*, 1992; Kieffer *et al.*, 1992) and well characterized by pharmacological studies (see review, Porreca *et al.*, 1995). The importance of δ-opioid receptors in the spinal cord of mice in mediating antinociception has been recognized (Mattia *et al.*, 1992; Tseng & Collins, 1993). Recent studies with an antisense oligodeoxynucleotide to δ-opioid receptor mRNA clearly confirmed previous pharmacological studies at the molecular level indicating a distinct δ-opioid receptor for antinociception in the spinal cord (Standifer 1994; Tseng & Collins, 1994; Tseng *et al.*, 1994; 1995; Narita & Tseng, 1995; Mizoguchi *et al.*, 1995; Narita *et al.*, 1996).

A limiting factor in the clinical utilization of opioids for pain relief is that repeated administration leads to the development of tolerance to and physical dependence on opioids. At the cellular level, tolerance can be viewed as a form of persistent receptor desensitization associated with repeated drug administration. Phosphorylation of opioid receptors by protein kinases, especially protein kinase C (PKC), is hypothesized to play a major role in this desensitization; hence, opioid receptors in tolerant and dependent states are thought to be highly phosphorylated (Narita *et al.*, 1994a,b,c; 1995; Mayer *et al.*, 1995; Mao *et al.*, 1995).

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Phorbol esters have a wide variety of pharmacological actions mediated through activation of PKC (Shearman *et al.*, 1989; Nishizuka, 1992; Yang & Tsien, 1993; Sasa *et al.*, 1995). Pei *et al.* (1995) have shown that a phorbol ester stimulates phosphorylation of the δ-opioid receptor in human embryonic kidney 293 cells, whereas a protein kinase A (PKA) activator, forskolin, had no such an effect. It is also proposed that PKC is involved in the functional uncoupling of the δ-opioid receptor from the inhibitory guanine nucleotide-binding protein (G_i) in striatal membranes of young guinea-pigs (Fukushima *et al.*, 1994) and in *Xenopus* oocytes (Ueda *et al.*, 1995). These findings indicate the possibility that PKC may be responsible for desensitization of the δ-opioid receptor-mediated antinociception. The aim of the present study was therefore to investigate the effects of a PKC activator, phorbol-12,13-dibutyrate (PDBu; Nishizuka, 1992; Yang & Tsien, 1993; Sasa *et al.*, 1995), on antinociception induced by intrathecal (i.t.) administration of a highly selective δ-opioid receptor agonist, [D-Ala^2] deltorphin II (Eraspamer *et al.*, 1989; Mattia *et al.*, 1992; Raynor *et al.*, 1994; Tseng & Collins, 1994; Narita & Tseng, 1995), in the mouse.

Sadée *et al.* (1994) have proposed that in the naive, a small fraction of opioid receptor (OR) is in the constitutively active state (OR*), and the conversion of OR to OR* is slow in the absence of an agonist. Upon treatment with an opioid agonist, there is an increased conversion of OR to OR* so that the dependent state is defined by an enhancement of OR* activity.

Based on their theories, tolerance results because fewer opioid receptors remain in the resting OR state. In order to test the hypothesis that opioid receptor desensitization involves a decrease in the number of the OR itself, we investigated the effects of *in vitro* treatment with PDBu on the specific binding of [^{3}H]-[D-Ser^2, Leu^5]enkephalin-Thr^6 (DSLET), a δ-opioid receptor agonist-sensitive site ligand, in the crude synaptic membrane of the mouse spinal cord.

Tolerance, or a reduced drug effect following chronic drug administration, develops to many opioid effects after prolonged treatment. We have previously found that a single i.t. injection of [D-Ala^2] deltorphin II produces an acute tolerance to the antinociceptive effect of a subsequent i.t. challenge of [D-Ala^2] deltorphin II in the mouse (Narita *et al.*, 1996). The present study was then designed to determine whether spinally PKC or PKA activation is specifically involved in the development of acute tolerance to the antinociception induced by i.t.-administered [D-Ala^2] deltorphin II in the mouse. For this purpose, we used a specific PKC inhibitor, calphostin C (Kobayashi *et al.*, 1989) and a highly selective PKA inhibitor, KT5720 (Kase *et al.*, 1987).

Methods

Animals

Male ICR mice weighing 23–27 g (SASCO, Inc., Omaha, NE) were used. Animals were housed 5 per cage in a room maintained at 22 ± 0.5°C with an alternating 12 h light-dark cycle. Food and water were available *ad libitum*. Animals were used only once.

Assessment of antinociception

Antinociception was determined by the tail-flick test (D'Amour & Smith, 1941). For measurement of the latency of the tail-flick response, mice were gently held by hand with their tail positioned in an apparatus (Model TF6, EMDIE Instrument Co., Maidens, VA) for radiant heat stimulation on the dorsal surface of the tail. The intensity of heat stimulus was adjusted so that the animal flicked its tail after 3 to 5 s. The inhibition of the tail-flick response was expressed as % maximum possible effect (% MPE) which was calculated as: $[T_1-T_0)/(T_2-T_0)] \times 100$, where T_0 and T_1 are the tail-flick latencies before and after the injection of opioid agonist and T_2 is the cut-off time which was set at 10 s for the tests to avoid injury of the tail.

Intrathecal injection

Intrathecal (i.t.) administration was performed following the method described by Hylden & Wilcox (1980) with a 10 μl Hamilton syringe with a 30 gauge needle. Injection volumes were 5 μl for i.t. injection.

Membrane preparation for receptor binding

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 volumes (w/v) of ice-cold 0.32 M sucrose with a Potter-Elvehjem tissue grinder. The homogenates were centrifuged at 4°C for 10 min at 1,000 *g*. The pellets were discarded and the supernatants were centrifuged at 4°C for 20 min at 20,000 *g* to obtain crude mitochondrial pellets. The mitochondrial pellets were resuspended in double-distilled deionized water and dispersed with a Potter-Elvehjem tissue grinder. The suspensions were centrifuged at 4°C for 20 min at 8,000 *g*. The pellets were discarded and the supernatant, including the soft buffy layer, was collected from each tube, incubated at 25°C for 2 h to degrade endogenous opioid ligands, and centrifuged at 4°C for 30 min at 40,000 *g* to obtain crude synaptosomal pellets. The crude synaptosomal pellets were resuspended in 50 mM Tris-HCl buffer (pH 7.4) and centrifuged at 4°C for 30 min at 40,000 *g*. The final pellets were stored at −70°C until experiments.

Opioid receptor binding assay

Just before the binding experiment, the stocked pellets were resuspended in 50 mM Tris-HCl buffer (pH 7.4) and centrifuged at 4°C for 30 min at 40,000 *g*. The pellets were resuspended in 50 mM Tris-HCl buffer (pH 7.4) and used for the binding assay. Binding assays for the δ-opioid receptor agonist-sensitive site were carried out in triplicate with [tyrosyl-3,5-^{3}H(N)]-Tyr-D-Ser-Gly-Phe-Leu-Thr ([^{3}H]-DSLET; 57.0 Ci mmol^{-1}; NEN, Boston, MA) at 4 nM in a final volume of 1.0 ml which contained 50 mM Tris-HCl buffer (pH 7.4) and 0.1 ml of the homogenated membrane fraction. In the case of the determination of the density and affinity of binding sites, [^{3}H]-DSLET binding was carried out in triplicate with final [^{3}H]-DSLET concentrations ranging 0.1 to 10 nM. The amount of membrane protein used in each assay was in the range of 50 to 150 μg, as determined by the method of Lowry *et al.* (1951). The test tubes were incubated for 120 min at 25°C. The specific binding was defined as the difference in binding observed in the absence and presence of 10^{-5} M unlabeled DSLET. Unlabelled Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol (DAMGO; 10 nM) was included in incubations containing [^{3}H]-DSLET to block binding of this radioligand to μ-opioid receptors. In order to investigate the effect of PDBu on [^{3}H]-DSLET binding, PDBu at different concentrations (0.03 to 10 μM) was added to the assay tube. The incubations were terminated by collecting the membranes on Whatman GF/B filters with a Brandel cell harvester (Model M-24, Brandel, MD). The filters were then washed three times with 5 ml Tris-HCl buffer (pH 7.4) at 4°C and transferred to scintillation vials. Then, 0.5 ml of Soluene-350 (Packard Instrument Company, Inc., Meriden, CT) and 5 ml of Hionic Fluor Cocktail (Packard Instrument Company) were added to the vials. After a 12 h equilibration period, the radioactivity in the samples was determined in a liquid scintillation analyzer (Model 1600 CA, Packard Instrument Company). Values for Scatchard analysis represent the mean ± s.e.mean of five independent determinations. Each independent set for Scatchard analysis contained 20 mice.

Drugs

The drugs used in the present studies were: phorbol-12,13-dibutyrate (PDBu; Research Biochemicals International, Natick, MA), calphostin C (Calbiochem-Novabiochem International, San Diego, CA), KT5720 ((8**R**, 9**S**, 11**S**)-(-)-9-hydroxy-9-n-hexyloxy-carbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo[a,g]cycloocta[cde]trinden-1-one, Calbiochem-Novabiochem International), [D-Ala^2,NMePhe^4, Gly(ol)^5]enkephalin (DAMGO; Peninsula Laboratory, Belmont, CA) and Tyr-D-Ser-Gly-Phe-Leu-Thr ([D-Ser^2, Leu^5]enkephalin-Thr^6: DSLET; Peninsula Laboratory). Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 ([D-Ala^2] deltorphin II) was synthesized by Dr John Richard (Molecular Research Laboratories, Durham, NC). In *in vivo* experiments, PDBu was dissolved in 0.01% ethanol in 0.9% sterile sodium chloride solution (saline). Calphostin C and KT5720 were dissolved in dimethyl sulphoxide 0.5% in saline. The doses of protein kinase inhibitors were chosen based on the data that they selectively block the respective protein kinases (Kase *et al.*, 1987; Kobayashi *et al.*, 1989; Narita *et al.*, 1995). Peptides were dissolved in saline containing 0.01% Triton X 100. The doses of the drugs showed no vehicle effect compared with that of saline.

Statistical analysis

The data are expressed as means ± s.e.mean. The relative potency (shift ratio) shown was calculated by the distance between parallel linear regression lines by use of a computer

programme (Tallarida & Murray, 1987). The binding data for the determination of the density (B_{max}) and affinity (K_d) of binding sites were evaluated by a computer-assisted analysis, EDBA and LIGAND (Biosoft, Cambridge, UK). Student's t test was used for the statistical analysis of B_{max} and K_d values. Comparisons of all other data were performed by ANOVA followed by Newman-Keuls's test (Tallarida & Murray, 1987).

Results

Effects of i.t. pretreatment with PDBu on antinociception induced by i.t.-administered [D-Ala²]deltorphin II

Groups of mice were pretreated i.t. with different doses (10 or 50 pmol) of PDBu or saline 30 min before an i.t. challenge with [D-Ala²]deltorphin II or saline. The tail-flick response was measured 10, 20, 30 and 60 min after the i.t. injection of [D-Ala²]deltorphin II (6.4 nmol) or saline (5 μl). PDBu at 10 and 50 pmol alone given i.t. did not affect the base line tail-flick latencies (Table 1). Pretreatment (i.t.) with PDBu for 30 min attenuated in a dose-dependent manner the inhibition of the tail-flick response induced by [D-Ala²]deltorphin II (Figure 1).

Table 1 Lack of effect of phorbol 12, 13-dibutyrate (PDBu) and calphostin C given i.t. on the tail-flick response latency

Time after injection (min)	*Tail-flick latencies (s)* 0	10	30
Saline ($n=26$)	3.7±0.2	3.7±0.2	3.6±0.1
PDBu 10 pmol ($n=15$)	3.2±0.2	3.7±0.2	3.4±0.3
PDBu 50 pmol ($n=15$)	3.5±0.3	3.5±0.2	3.6±0.2
Calphostin C 1 pmol ($n=10$)	4.1±0.3	3.8±0.2	3.9±0.3
Calphostin C 10 pmol ($n=10$)	3.8±0.3	3.7±0.2	3.6±0.2

Groups of mice were treated i.t. with PDBu (10 or 50 pmol), calphostin C (1 or 10 pmol) or saline. The tail-flick responses were measured just before (0 min), and 10 and 30 min after the injection.

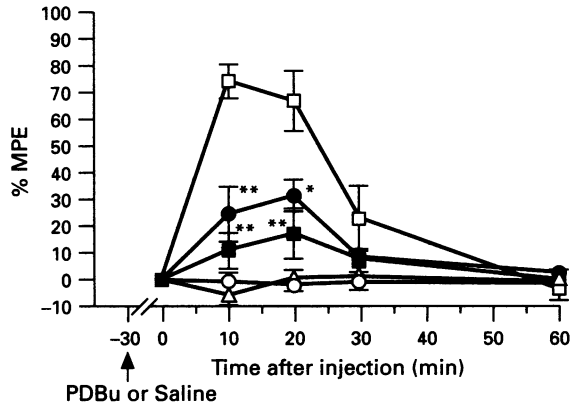

Figure 1 Time courses of antinociception induced by i.t.-administered [D-Ala²]-deltorphin II in mice receiving i.t. phorbol 12,13-dibutyrate (PDBu): (○) 5 μl saline + saline; (□) saline + 6.4 nmol [D-Ala²]-deltorphin II; (△) 50 pmol PDBu + saline; (●) 10 pmol PDBu + 6.4 nmol [D-Ala²]-deltorphin II; (■) 50 pmol PDBu + 6.4 nmol [D-Ala²]-deltorphin II. Groups of mice were pretreated i.t. with PDBu or saline 30 min before an i.t. challenge with [D-Ala²]-deltorphin II or saline. The tail-flick response was performed 10, 20, 30 and 60 min after [D-Ala²]-deltorphin II or saline injection. The vertical lines represent s.e.mean; $n=7-10$ mice for each group. $^{**}P<0.01$, compared with saline + [D-Ala²]-deltorphin II.

Effect of i.t. pretreatment with PDBu on the dose-response curve for antinociception induced by i.t.-administered [D-Ala²]deltorphin II

As shown in Figure 2, [D-Ala²]deltorphin II at doses of 0.6 to 12.8 nmol given i.t. caused a dose-dependent inhibition of the tail-flick response in mice pretreated i.t. with saline for 30 min. Pretreatment with PDBu (50 pmol, i.t.) 30 min prior to an i.t. challenge with [D-Ala²]deltorphin II attenuated the inhibition of the tail-flick response induced by i.t.-administered [D-Ala²]deltorphin II; the dose-response curve for [D-Ala²]-deltorphin II antinociception was markedly shifted to right by 5.0 (lower: 3.1; upper: 7.9) fold.

Effects of calphostin C on the PDBu-induced attenuation of the tail-flick inhibition induced by i.t.-administered [D-Ala²] deltorphin II

Groups of mice were pretreated i.t. with saline, PDBu (10 or 50 pmol) alone or a combination of PDBu (50 pmol) and calphostin C (specific PKC inhibitor; 1 or 10 pmol) 30 min before challenge with [D-Ala²]deltorphin II (6.4 nmol, i.t.). The tail-flick response was measured 10 min after the [D-Ala²]-deltorphin II challenge. Pretreatment with PDBu for 30 min attenuated the [D-Ala²]deltorphin II-induced inhibition of the tail-flick response. The attenuation of [D-Ala²]deltorphin II-induced tail-flick inhibition by PDBu (50 pmol) was reversed in a dose-dependent manner by concomitant pretreatment with calphostin C (Figure 3). I.t. treatment with calphostin C alone did not affect the base line tail-flick latencies (Table 1).

Effects of KT5720 and calphostin C on the i.t.-administered [D-Ala²]deltorphin II-induced antinociception and the development of acute tolerance to the i.t. [D-Ala²deltorphin II antinociception

Concomitant i.t injection of a specific PKC inhibitor, calphostin C (1.3, 5.1 or 12.7 pmol) or a selective PKA inhibitor, KT5720 (7.4 or 18.6 pmol) did not have any effect on antinociception induced by i.t.-administered [D-Ala²]deltorphin II (Table 2). Other groups of mice were pretreated i.t. with saline (5 μl), [D-Ala²]deltorphin II (6.4 nmol) alone or a combination of [D-Ala²]deltorphin II (6.4 nmol) and calphostin C (1.3, 5.1 or 12.7 pmol) or KT5720 (7.4 or 18.6 pmol) 3 h before a subsequent i.t. challenge of [D-Ala²]deltorphin II (6.4 nmol). An i.t. pretreatment with [D-Ala²]deltorphin II markedly attenuated the antinociceptive effect induced by the second i.t. injection of [D-Ala²]deltorphin II (61.4% inhibition). The attenuation of [D-Ala²]deltorphin II-induced antinociception produced by [D-Ala²]deltorphin II pretreatment was dose-dependently blocked

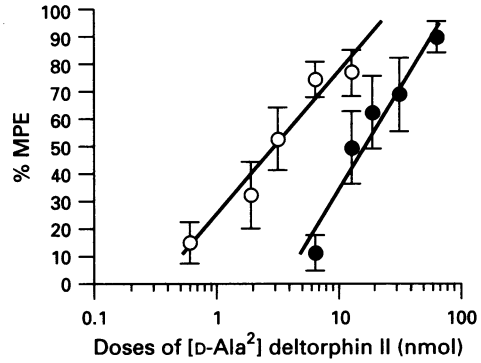

Figure 2 Effects of i.t. pretreatment with phorbol 12,13 dibutyrate (PDBu) on the dose-response curve for antinociception by i.t.-administered [D-Ala²]-deltorphin II. Groups of mice were pretreated i.t. with 50 pmol PDBu (●) or saline (○) 30 min before an i.t. challenge with [D-Ala²]-deltorphin II. The tail-flick responses were measured 10 min after an i.t. injection of [D-Ala²]-deltorphin II. The vertical lines represent s.e.mean; $n=7-10$ mice for each group.

by i.t. pretreatment with calphostin C (Table 2). On the other hand, KT5720 given concomitantly with [D-Ala^2]deltorphin II had no effect on the development of acute tolerance to [D-Ala^2]deltorphin II antinociception (Table 2).

Effects of PDBu on the specific binding of [^{3}H]-DSLET to membranes of the mouse spinal cord

The crude synaptic membranes of spinal cord were incubated with a range of PDBu concentrations (0.03 to 10 μM) for 2 h at 25°C. As shown in Figure 4, the specific δ-opioid receptor binding of 4 nM [^{3}H]-DSLET to membranes of the mouse spinal cord was dose-dependently decreased by treatment with PDBu. Binding was significantly reduced to 85.8–67.0% of the non-pretreatment group in the range of 1 to 10 μM. The combination of calphostin C 100 nM with 10 μM PDBu significantly reversed the decrease of [^{3}H]-DSLET binding induced by PDBu (data not shown).

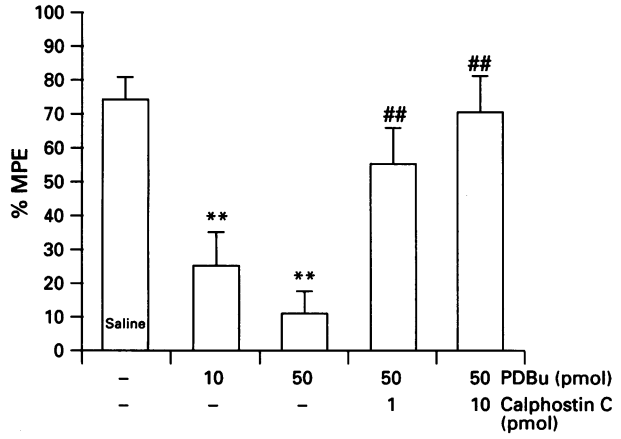

Figure 3 Effects of calphostin C on the phorbol 12,13-dibutyrate (PDBu)-induced attenuation of the tail-flick inhibition induced by i.t.-administered [D-Ala^2]-deltorphin II. Groups of mice were pretreated i.t. with saline (5 μl), PDBu (10 or 50 pmol) alone or a combination of PDBu (50 pmol) and calphostin C (1 or 10 pmol) 30 min before i.t. challenge with [D-Ala^2]-deltorphin II (6.4 nmol). The tail-flick response was measured 10 min after a challenge with [D-Ala^2]-deltorphin II. The vertical line represents s.e.mean; $n=7-10$ mice for each group. $^{**}P<0.01$, compared with saline pretreatment. $^{\#\#}$ $P<0.01$, compared with PDBu (50 pmol) pretreatment alone.

Scatchard analysis was performed on membranes treated with 10 μM PDBu. Figure 5 illustrates Scatchard plots for control and PDBu-treated membrane fractions, indicating the presence of a single class of binding sites. A mean B_{max} value of 83.08±2.13 fmol mg^{-1} protein with a K_d of 2.66±0.25 nM was found on untreated membranes. PDBu (10 μM)-treated membranes displayed a 30.7% reduction in the number of [^{3}H]-DSLET binding sites (57.58±3.70 fmol mg^{-1} protein; $P<0.01$, as compared to untreated group) with no significant change in affinity (2.46±0.29 nM).

Discussion

PKC functions as a critical component of the signal transduction pathways that cells utilize to recognize and respond to a variety of extracellular agents (Nishizuka, 1986; 1988; 1992; Shearman *et al.*, 1989). These external stimuli increase the level of diacylglycerol (DAG); DAG then functions as a second messenger by binding to the regulatory domain of PKC and activating PKC. Phorbol esters, such as PDBu, are the most potent known activators of PKC. Phorbol esters can activate PKC apparently by binding to the regulatory domain of PKC (Akita *et al.*, 1990; Nishizuka, 1992; Sasa *et al.*, 1995). The effects of phorbol esters on a variety of cell membrane receptor functions have been examined and correlated with their ability to stimulate PKC activity (Bazzi & Nelsestuen, 1989; Akita *et al.*, 1990). We found in the present study that i.t. pretreatment of mice with PDBu at low doses, which injected alone did not have any effect on the nociceptive threshold of the tail-flick response, caused a dose-dependent attenuation of the anti-

Table 2 Effects of specific protein kinase A (PKA) and PKC inhibitors on i.t.-administered [D-Ala^2]deltorphin II-induced antinociception and the development of acute tolerance to i.t.-administered [D-Ala^2]deltorphin II-induced antinociception

First injection	*Second injection challenge*	*%MPE*
[D-Ala^2]deltorphin II	None	79.7±6.5
[D-Ala^2]deltorphin II + calphostin C (1.3 pmol)	None	69.5±9.5
[D-Ala^2]deltorphin II + calphostin C (5.1 pmol)	None	74.2±7.4
[D-Ala^2]deltorphin II + calphostin (12.7 pmol)	None	71.0±6.5
[D-Ala^2]deltorphin II + KT5720 (7.4 pmol)	None	77.0±9.0
[D-Ala^2]deltorphin II + KT5720 (18.6 pmol)	None	77.6±10.0
[D-Ala^2]deltorphin II	[D-Ala^2]deltorphin II	32.2±6.3**
[D-Ala^2]deltorphin II + calphostin C (1.3 pmol)	[D-Ala^2]deltorphin II	51.8±11.5
[D-Ala^2]deltorphin II + calphostin C (5.1 pmol)	[D-Ala^2]deltorphin II	63.2±10.7
[D-Ala^2]deltorphin II + calphostin C (12.7 pmol)	[D-Ala^2]deltorphin II	68.9±7.2#
[D-Ala^2]deltorphin II + KT5720 (7.4 pmol)	[D-Ala^2]deltorphin II	33.7±7.2**
[D-Ala^2]deltorphin II + KT5720 (18.6 pmol)	[D-Ala^2]deltorphin II	30.1±10.6**

Calphostin C and KT5720 are specific PKC and PKA inhibitors, respectively. All drugs were injected intrathecally in mice. Calphostin C or KT5720 was concomitantly injected i.t. with [D-Ala^2]deltorphin II (6.4 nmol). The second i.t. injection of [D-Ala^2]deltorphin II (6.4 nmol) was performed 3 h after the first injection. The tail-flick latency was measured 10 min after the first or second injection. The values represent the mean±s.e.mean of % maximum possible effect (MPE); $n=9-17$ mice for each group. $^{**}P<0.01$, compared to mice with a single i.t. injection of [D-Ala^2]deltorphin II. $^{\#}P<0.05$, compared to mice injected with [D-Ala^2]deltorphin II + [D-Ala^2]deltorphin II.

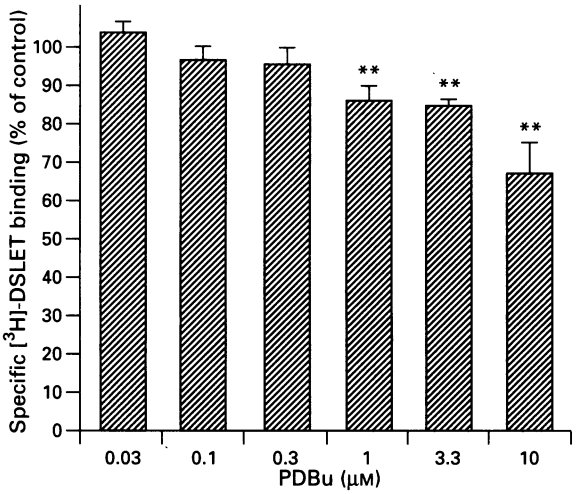

Figure 4 Effect of phorbol 12,13-dibutyrate (PDBu) on the specific binding of [^{3}H]-[D-Ser^2, Leu^5]enkephalin-Thr^6 ([^{3}H]-DSLET) to membranes of the mouse spinal cord. The δ-opioid receptor binding assays were carried out in triplicate with [^{3}H]-DSLET at 4 nM. The assay tubes were incubated for 2 h at 25°C with or without PDBu at different concentrations (0.03 to 10 μM). The specific binding was defined as the difference in binding observed in the absence and presence of 10^{-5} M unlabelled DSLET. Unlabelled [D-Ala^2, NMePhe^4, Gly(ol)^5]enkephalin (DAMGO, 10 nM) was included in incubations containing [^{3}H]-DSLET to block binding of this radioligand to μ-opioid receptors. [^{3}H]-DSLET binding was determined in each in more than 3 sets. Each set contained more than 5 mice. The vertical line represents s.e.mean. $^{**}P<0.01$, compared with non-treatment (100%).

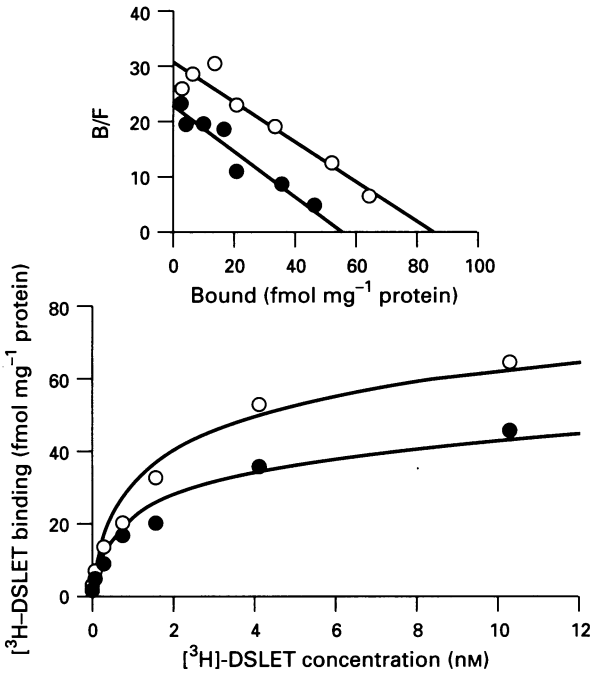

Figure 5 Saturation curve and Scatchard plot (inset) of the effect of phorbol 12,13-dibutyrate (PDBu, 10 μM) on [^{3}H]-DSLET binding to membranes of the mouse spinal cord: (○) non-treatment; (●) 10 μM PDBu. [^{3}H]-DSLET binding was carried out in triplicate with final [^{3}H]-DSLET concentrations ranging from 0.1 to 10 nM. The assay tubes were incubated for 2 h at 25°C with or without PDBu at 10 μM. The specific binding was defined as the difference in binding observed in the absence and presence of 10^{-5} M unlabelled DSLET. Unlabelled DAMGO (10 nM) was included in incubations containing [^{3}H]-DSLET. A representative experiment that was replicated five times is shown. For key to abbreviations used see legend of Figure 4.

nociceptive effect induced by i.t.-administered δ-opioid receptor agonist [D-Ala^2]deltorphin II. The attenuation by PDBu of δ-opioid receptor agonist-induced antinociception appears to be specifically mediated by the activation of PKC. This contention is supported by the finding that attenuation of [D-Ala^2]deltorphin II-induced antinociception by PDBu was blocked by concomitant pretreatment with a specific PKC inhibitor, calphostin C. Calphostin C and related compounds have been isolated from a fungus *Cladosporium cladosporioides* (Kobayashi *et al.*, 1989a). Calphostin C specifically inhibits DAG binding to the regulatory domain of PKC and therefore is a more selective inhibitor than staurosporine or 1-(5-isoquinolinyl-sulphonyl)-2-methylpiperazine (H-7), which interact with the catalytic domain (ATP-binding site) of PKC that shares substantial homology with other protein kinases. The result provides evidence that i.t.-administered PDBu-induced attenuation of spinal δ-opioid receptor-mediated antinociception is specifically mediated by the activation of PKC in the spinal cord.

PKC has been found to exist in both the cytosolic and membrane fractions (Nishizuka, 1992). Modulation of specific membrane receptors by phorbol esters appears to be one of the early biological responses to these compounds. Activated PKC by phorbol esters can act on membrane receptors, or other membrane components, to influence receptor-mediated events such as receptor-second messenger coupling (Shahabi & Sharp, 1993; Fukushima *et al.*, 1994; Ueda *et al.*, 1995). We found in the present *in vitro* binding study that treatment with PDBu caused a dose-dependent decrease of [^{3}H]-DSLET binding in membranes of the mouse spinal cord. This effect was reversed by calphostin C. In our preliminary studies, we found that DSLET at 10 μM did not interfere acutely with 15 nM [^{3}H]-PDBu binding in the crude synaptic membrane of mouse spinal cord under the same conditions as in the present study. Additionally, phosphatidylserine (PS), which is a phospholipid and can activate PKC by either Ca^{2+}-dependent or -independent mechanisms (Hunnun *et al.*, 1985), also inhibited [^{3}H]-DSLET binding in membranes (unpublished observation). It is, therefore, unlikely that PDBu caused a simple displacement of [^{3}H]-DSLET binding on membranes.

Based on Scatchard analysis, PDBu displayed a marked reduction in the B_{max} value for [^{3}H]-DSLET binding with no significant change in the K_d value. These findings suggest that the activation of membrane-bound PKC by PDBu leads to a decrease in the number of spinal δ-opioid receptor agonist-sensitive binding sites in the mouse. Taken together with the present *in vivo* results, it is possible that the PDBu-induced antinociceptive unresponsiveness to δ-opioid receptor agonist is due to a loss of specific δ-agonist binding by activated PKC.

A single i.t injection of [D-Ala^2]deltorphin II produced acute antinociceptive tolerance to a second i.t. challenge of [D-Ala^2]deltorphin II. This tolerance to i.t. [D-Ala^2]deltorphin II-induced antinociception occurred rapidly; the effect became apparent within 3 h after the first injection. The present data are supported by the findings that desensitization to opioid-induced pharmacological actions is rapidly induced by single *in vivo* or *in vitro* treatment with opioids (Nomura *et al.*, 1994; Ueda *et al.*, 1995; Narita *et al.*, 1996). This acute tolerance to [D-Ala^2]deltorphin II antinociception is reversible and recovers in 24 h (Narita *et al.*, 1996). A similar time course was also found for the [^{3}H]-DSLET binding studies. Pretreatment of mice i.t. with [D-Ala^2]deltorphin II significantly decreased [^{3}H]-DSLET binding 3 h after treatment, but levels had returned to control by 24 h after injection (Narita *et al.*, 1996; unpublished observation). We found in the present study that calphostin C, at low doses, which injected alone had no effect on antinociception induced by i.t. injection of [D-Ala^2]deltorphin II, caused a dose-dependent blockade of the development of tolerance to i.t.-administered [D-Ala^2]deltorphin II-induced antinociception. These results suggest that activated PKC, which rapidly decreases the δ-opioid receptor agonist-sensitive site, is involved in the development of acute antinociceptive tolerance to [D-Ala^2]deltorphin II.

The acute tolerance study indicates that agonist-induced desensitization of δ-opioid receptors caused by the activation

of PKC is a key event initiating 'homologous receptor desensitization' in the mouse spinal cord. This contention is supported by the recent finding that δ-opioid receptors mediate phospholipase C (PLC) activation through G_i in functional reconstitution experiments in *Xenopus* oocytes (Miyamae *et al.*, 1993; Ueda *et al.*, 1994). The function of such an intracellular negative feed back system by PKC is to maintain a homeostatic state. Thus the stimulation of δ-opioid receptors by a δ-opioid receptor agonist desensitizes the δ-opioid receptor for antinociception in order to maintain a basal level of pain sensitivity.

It has been hypothesized that prolonged stimulation of an opioid receptor by an agonist results in a slow conversion of the opioid receptor to a constitutively active state (OR*) (Sadée *et al.*, 1994). The resting state of the opioid receptor (OR), which can be activated by the receptor agonist, enters a slow equilibrium with the agonist-independent OR*, which represents a highly phosphorylated state. Based on recent cloning studies, a number of potential phosphorylation sites by protein kinases are present in cloned δ-opioid receptors (Evans *et al.*, 1992; Miotto *et al.*, 1995). Indeed, phorbol esters stimulate phosphorylation of the δ-opioid receptor in human embryonic kidney 293 cells (Pei *et al*, 1995). It is possible that the phosphorylation of δ-opioid receptors by PKC causes the apparent decrease in the number of δ-opioid receptor agonist-sensitive sites: (1) by rendering receptors less capable or unable to bind ligand due to an alternation in intrinsic physical state or (2) by altering ligand-receptor binding due to a change in receptor-receptor interactions that determines the equilibrium between monomeric vs. dimeric forms in the membrane (Downward *et al.*, 1985; Shahabi & Sharp, 1993). Our results, therefore give support to the possibility that phosphorylation of δ-opioid receptors induced by activation of PKC is the mechanism for the desensitization of spinal δ-opioid receptor functions.

Acute treatment with opioids inhibits adenylate cyclase and thus lowers cyclic AMP levels (Nestler, 1992). In contrast, chronic treatment with a prototype of the μ-opioid receptor agonist, morphine, increases PKA activity in the locus coeruleus, but not in several other brain regions (Nestler & Tallman, 1988). Our present data show that, in contrast to the results with calphostin C-induced responses, the PKA inhibitor, KT5720, did not have any effect on the development of acute tolerance to [D-Ala^2]deltorphin II-induced antinociception. KT5720, isolated from *Nocardiopsis sp.*, has been found to inhibit PKA selectively (Kase *et al.*, 1987). It seems unlikely that PKA activity is increased during short-term exposure to opioid agonists. Thus, PKA appears not to be involved in acute tolerance to antinocicpetion induced by i.t.-administered δ-opioid receptor agonists.

In the study with the chronic animal model of morphine tolerance/dependence, PKC activity has been demonstrated to be up-regulated with a time course that closely paralleled the development of antinociceptive tolerance to morphine (Narita *et al.*, 1994b,c; Mayer *et al.*, 1995; Mao *et al.*, 1995). More recently, we found that inhibition of PKC blocked the development of acute antinociceptive tolerance to the i.t.-administered selective μ-opioid receptor agonist DAMGO (Narita *et al.*, 1995). From our results together with these observations, we hypothesize that PKC is involved in the development of opioid tolerance/dependence.

In conclusion, we have demonstrated that i.t. pretreatment of mice with PDBu attenuates the antinociception induced by the i.t.-administered δ-opioid receptor agonist [D-Ala^2]deltorphin II. The effects of PDBu were specifically due to the activation of PKC because a highly selective PKC inhibitor calphostin C antagonized these effects. In the binding studies, treatment with PDBu decreased the δ-opioid receptor agonist-sensitive binding sites in the membrane of the mouse spinal cord. Furthermore, concomitant pretreatment of mice with calphostin C prevented the development of acute tolerance to antinociception induced by i.t.-administered [D-Ala^2]deltorphin II, whereas a highly selective PKA inhibitor, KT5720, did not have any effect on the development of acute tolerance to [D-Ala^2]deltorphin II antinociception. Our results suggest that activation of PKC, but not PKA, is involved in the process of homologous desensitization of spinal δ-opioid receptor-mediated antinociception in the mouse.

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