Protein Kinase C-Mediated Acute Tolerance to Peripheral $\mu$-Opioid Analgesia in the Bradykinin-Nociception Test in Mice

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ABSTRACT

We studied the acute tolerance liability of peripheral opioid analgesia in mice. The analgesia was assessed by the inhibition of bradykinin (BK)-induced nociceptive action by using a newly developed flexor reflex paradigm. Morphine [intraplantarly (i.pl.)] given ipsilaterally to BK showed a dose-dependent reduction of the BK (2 pmol) responses, whereas the administration of 10 nmol of morphine into the contralateral side failed to show any significant analgesic effects. Furthermore, DAMGO ($\mu$-opioid receptor (MOR) agonist), and U-69593, a $\delta$-opioid receptor (KOR) agonist, but not DSLET ($\kappa$-opioid receptor (KOR)) agonist, showed similar analgesia on the BK responses. The morphine- or U-69593 ([5c7a,8β]-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8y]-benzeneacetamide)-induced analgesia was markedly attenuated by the intrathecal injection of each antisense oligodeoxynucleotide for the MOR or KOR, respectively, suggesting that these peripheral analgesia are mediated through MORs and KORs located on nociceptor endings, respectively. As BK response was completely recovered to the control level 4 h after morphine (3 nmol i.pl.) or U-69593 (10 nmol i.pl.) administration, these compounds were challenged again to see the inhibition of BK responses. Although morphine analgesia by the second challenge was markedly attenuated, U-69593 analgesia was not. The attenuated morphine analgesia was completely reversed by the pretreatment of calphostin C, Go6976, or HBDDE, a protein kinase C inhibitor, but not by KT-5720, a protein kinase A inhibitor. These results suggest that selective acute tolerance of peripheral morphine analgesia, but not U-69593 analgesia, through MORs and KORs located on polymodal nociceptors, respectively, in the bradykinin-nociception test in mice was mediated through protein C activation.

It is accepted that opioid analgesia is particularly related to the activation of opioid receptors within the central nervous system, such as periaqueductal gray matter (Hosobuchi et al., 1977), nucleus reticularis para-gigantocellularis (Takagi, 1980), nucleus raphe magnus (Dickenson et al., 1979), and dorsal horn of spinal cord (Yeung and Rudy, 1980). However, there are also accumulating reports that opioids exert potent peripheral analgesia (Khasar et al., 1996; Kolesnikov et al., 1996a,b; Zhou et al., 1998). Indeed, opioid receptors were found on peripheral nerve endings (nociceptor endings) of thinly myelinated (Aδ-fiber) and unmyelinated (C-fiber) sensory neurons in animals (Hassan et al., 1993; Stein, 1995), and significant amounts of opioid receptor mRNA were also found in the dorsal root ganglia (Schafer et al., 1995).

In many nociception tests to evaluate analgesic actions, various mechanical and thermal stimulations have been used. Although these stimulations are supposed to directly drive mechanothermal Aδ- and polymodal C-fiber nociceptors, they are also expected to drive indirect mechanisms, including a release of pain-producing substances such as bradykinin (BK), histamine, serotonin, ATP, and potassium ion (for a review, see Ueda, 1999). Therefore, it is difficult to identify or discuss the molecular basis of mechanism in pain production and analgesia. Recently, we developed a simple and sensitive peripheral nociception test in mice (Inoue et al., 1998a; Ueda, 1999), where we assessed the flexor responses in mice after the local administration of a pain-producing substance, such as BK or substance P. Because BK is known to stimulate C-fiber nociceptors, the analgesia evaluated as an inhibition of BK responses would be more likely attributed to the action on polymodal nociceptors. The fact that the cell body of sensory neurons is located in the distance from nociceptor endings gives some advantages to this paradigm of nociceptor endings gives some advantages to this paradigm of nociceptor endings gives some advantages to this paradigm of nociceptor endings.
nociception test, because the selective reduction of expression of specific molecules in nociceptors can be performed by intrathecal injection with antisense oligodeoxynucleotide (AS-ODN; Ueda, 1999) and the signaling mechanisms in nociceptor endings without effects on the cell body can be discussed.

Prolonged and repeated exposure to opioid agonists reduces the responsiveness of opioid receptors to its agonist over time. This loss of receptor function was hypothesized to contribute to the opiate tolerance, dependence, and addiction in humans (Nestler, 1992). Substantial experimental evidence has divided this loss of function into separate but related receptor events: 1) down-regulation, 2) desensitization, and 3) internalization. The mechanisms of opioid tolerance observed in cell culture studies have been well discussed, but little is known of such mechanisms in in vivo studies of opioid analgesia. Here, we demonstrate that some types of opioid receptors may be involved in the peripheral analgesia through an inhibition of BK stimulation of polymodal nociceptors in mice and the selective acute tolerance to the peripheral analgesia of morphine (a \( \mu \)-opioid agonist), but not U-69593-induced (a \( \kappa \)-opioid agonist) one through a protein kinase C (PKC)-mediated mechanisms.

**Materials and Methods**

**Animals.** Male ddY-strain mice weighing from 20 to 22 g were kept in a room maintained at 21 ± 2°C with free access to a standard laboratory diet (MF; Oriental Yeast, Tokyo, Japan) and tap water. Procedures were approved by the Nagasaki University Animal Care Committee and amplified with the recommendations of the International Association for the Study of Pain (Zimmermann, 1983).

**Drugs.** The drugs used were morphine (obtained from Takeda Chemical Industries, Osaka, Japan); DAMGO, DSLET, U-69593, naloxone, d-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-amide (CTOP), nor-binaltorphimine (nor-BNI), and BK (all obtained from Sigma, St. Louis, MO); calphostin C and KT-5720 (obtained from Kyowa Medics, Tokyo, Japan); and Go6976, HBDDE, and Rottlerin (obtained from Calbiochem, La Jolla, CA). All drugs except for U-69593, calphostin C, KT5720, Go6976, HBDDE, and Rottlerin were dissolved in physiological saline. U-69593 was dissolved in 1.5% ethanol. Calphostin C, KT-5720, Go6976, HBDDE, and Rottlerin were dissolved in 30% dimethyl sulfoxide. In many experiments, drugs except for calphostin C and KT-5720 were given by intraplantar (i.pl.) injection in a volume of 2 \( \mu \)l. Calphostin C, KT-5720, Go6976, HBDDE, and Rottlerin were given by i.pl. injection in a volume of 5 \( \mu \)l. In some experiments, drugs were given in a large volume (0.1 ml/10 g b.wt.) to the back (s.c.). The AS-ODN (5′-GCC GGC GCT GCT GTC CAT-3′) and its missense oligodeoxynucleotide (MS-ODN; 5′-GCC GGC GCT GCT GTC CAT-3′) for \( \mu \)-opioid receptor (MOR; Min et al., 1994) and the AS-ODN (5′-GTT GCC TCC AAG GAC TAT GCC-3′) and its MS-ODN (5′-GGG TCC CTA AAG GCA TGC TGC-3′) for \( \kappa \)-opioid receptor (KOR; Chien et al., 1994), were synthesized, freshly dissolved in physiological saline, and used for intrathecal (i.t.) injection according to the protocol of Hylden and Wilcox (1980) in a volume of 2 \( \mu \)l on the 1st, 3rd, and 5th days. On the 6th day, mice were used for the nociception test, because we used light and soft polyethylene cannulae, they did not fall off the paw during the experiments. All experiments were started after the complete recovery of mouse from the light ether anesthesia (20–30 min) and the confirmation that the i.pl. injection of saline did not show any significant flexor responses. BK was given i.pl. at 10 and 5 min before and 5, 10, 20, and 30 min after opioid or vehicle injection. In most experiments, the results were expressed as percent analgesia, using the following equation: (1 – BK response (millimeters) after test drug administration/the average of twice control BK responses) × 100 (%). In some experiments, analgesia was also evaluated by the area under the analgesic curve (AUC) obtained by plotting analgesia (%) on the ordinate, and time after morphine (i.pl.) administration (minutes) on the abscissa. In this case, morphine analgesia was assessed by percentage of the maximal AUC, which represents the analgesia when BK response is completely inhibited during periods from 5 to 30 min after drug injection. Thus, the maximal AUC was calculated to be 2500 (%·min). The median analgesic dose (\( \text{AD}_{50} \)) was calculated from the linear regression curve of the percentage of maximal against log dose of opioid. To get S.E.M., we carried out five separate experiments, in which three different doses of morphine were tested.

**Western Blot Analysis.** SDS-polyacrylamide gel electrophoresis was performed as described (Yoshida and Ueda, 1999). Visualization of immunoreactive bands was performed by using an enhanced chemiluminescent substrate for detection of horseradish peroxidase, Super Signaling Substrate (Pierce Chemical Co., Rockford, IL). The intensities of immunoreactive bands were analyzed by NIH Image after scanning exposed films.

**Statistical Analysis.** The data were analyzed using Student’s \( t \) test after multiple comparisons of the ANOVA. The criterion of significance was set at \( P < .05 \). All results are expressed as the mean ± S.E.

**Results**

**Dose-Dependent Analgesia by Local Application of Morphine.** The local application of BK at 2 pmol into the planta of hind limb (i.pl.) produced a nociceptive flexor response, and there were stable responses in amplitude on successive tests. The i.pl. injection of saline did not show any significant flexor responses. BK in ranges of 0.02 to 20 pmol (i.pl.) showed such responses in a dose-dependent manner and the average of nociceptive dose (± S.E.M.) showing 50% of maximal reflex was 0.71 ± 0.09 pmol (n = 5). The BK (2 pmol)-induced responses were completely abolished by the i.pl. injection of B_2-type BK receptor antagonist (Inoue et al., 1997).

We studied the peripheral analgesia of morphine on such BK-induced flexor responses. When the i.pl. injection of 1 nmol of morphine was given 5 min after the second BK challenge, the following BK-induced nociceptive responses were rapidly attenuated and abolished 20 min after the mor-
phine challenge (Fig. 1A); however, they were completely recovered 180 min after the morphine challenge (data not shown). Such morphine “analgesia” was observed in a dose-dependent manner in ranges of 100 pmol to 1 nmol (Fig. 1B) throughout experiments within 30 min. The dose dependence was also observed when the analgesia was expressed as a percentage of maximal AUC for 30 min (Fig. 1C), and the AD$_{50}$ value was 274 ± 23 pmol ($n = 5$).

**Peripheral Morphine Analgesia.** When a higher amount of morphine at 10 nmol, which corresponds to 0.18 mg/kg, was injected locally into the contralateral side of hind limb to the BK injection side or systemically into the s.c. space of the back, there was no significant suppression of BK responses for 30 min (Fig. 2A). On the other hand, the morphine analgesia by i.pl. injection was abolished by the ipsilateral injection of naloxone (1 nmol), but not by the contralateral one (Fig. 2B).

**Opioid Receptor Type-Specific Peripheral Analgesia.** DAMGO, a specific MOR agonist, at 1 nmol (i.pl.) showed a potent analgesia by suppressing BK responses for 30 min (Fig. 3A). The analgesia by DAMGO lasts for 120 min. The AD$_{50}$ was $33 \pm 4$ pmol ($n = 5$) for DAMGO (Table 1), and this value was 8 times lower than that for morphine (274 pmol). On the other hand, 3 nmol of U-69593, a specific KOR agonist, showed analgesic effects equivalent to those of morphine at 1 nmol (Fig. 3A), and were also completely recovered after 180 min (data not shown). The AD$_{50}$ of U-69593 was $1045 \pm 102$ pmol ($n = 5$, Table 1). However, DSLET, a specific $\delta$-opioid receptor (DOR) agonist, at a dose up to 30 nmol showed no significant effects (Fig. 3A).

To confirm the opioid receptor type-specificity, the pretreatments with antagonists, such as naloxone (relatively $\mu$-type), CTOP (selectively $\mu$-type), and nor-BNI (selectively $\kappa$-type), were used. DAMGO and U-69593 showed a potent analgesia with $\mu$-opioid receptor type specificity, whereas DSLET showed no significant effects in this experiment. These results suggest that the analgesia induced by DAMGO was mediated by $\mu$-opioid receptor type, and that the analgesia induced by U-69593 was mediated by $\kappa$-opioid receptor type. However, DSLET showed no significant effects, indicating that the analgesia induced by DSLET was not mediated by the opioid receptor subtype used in this study.
k-type), were carried out. The analgesic effects of 1 nmol of DAMGO or 3 nmol of U-69593 were markedly blocked by equimoles of antagonists, respectively (Fig. 3B).

**TABLE 1**

Comparison of opioid subtypes analgesia on the bradykinin-flexor responses in mice

<table>
<thead>
<tr>
<th>Agonist</th>
<th>AD50 (nmol)</th>
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<tr>
<td>DAMGO (μ-opioid subtype)</td>
<td>0.033 ± 0.004</td>
</tr>
<tr>
<td>U-69593 (κ-opioid subtype)</td>
<td>1.045 ± 0.102</td>
</tr>
<tr>
<td>DSLET (δ-opioid subtype)</td>
<td>&gt;30</td>
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</table>

The AD50 was calculated from the linear regression curve of the percentage of maximal AUC, against log doses of opioid. Results are given as the mean ± S.E. from five separate experiments. The details are described in Materials and Methods.

**Development of Acute Tolerance on Morphine-Peripheral Analgesia.** To evaluate the development of peripheral acute tolerance by morphine pretreatment, mice were given 3 nmol of morphine (i.pl.), a dose 3 times higher than the maximal dose (1 nmol) for peripheral analgesia. When the nociceptive response by BK (2 pmol) was assessed at different times after the first morphine treatment, the time-dependent recovery was observed between 1 and 4 h (Fig. 6A). Because the BK response at 4 h after morphine treat-
ment was equivalent to that with vehicle treatment, we decided to use this morphine pretreatment and assess the analgesic action by the second morphine given 4 h after the first challenge for additional experiments. As shown in Fig. 6B, the morphine (3 nmol i.pl.) injection to mice pretreated with vehicle showed a time course of analgesia similar to that of 1 nmol of morphine in naive mice, as seen in Fig. 1B. However, there was a marked decrease in the analgesia by 3-nmol morphine in morphine (3 nmol)-pretreated mice (Fig. 6B). When various doses of morphine were given to assess the analgesic activity in morphine-pretreated mice, the dose-dependent analgesia was observed while the dose range was shifted to a higher one than that in naive mice (Fig. 6C). The AD_{50} of morphine in the pretreated mice became 10 nmol, a 30-times-higher dose than that (0.3 nmol) in naive mice. There was no change in U-69593 analgesia in morphine-pretreated mice (Fig. 6D); therefore, we suppose that the acute tolerance to morphine is due to the functional change at the receptor level.

PKC Involvement in the Acute Tolerance of Morphine-Induced Peripheral Analgesia. To characterize the mechanism of acute tolerance of peripheral analgesia, we assessed the effects of protein kinase inhibitors on it. When mice were pretreated with calphostin C, a PKC inhibitor or KT-5720, a cyclic AMP-dependent protein kinase (PKA) inhibitor, there was no significant change in the BK-induced nociceptive activity 4 h after such inhibitor injection, compared with vehicle-treated mice (Fig. 7A), although the BK responses were partially inhibited 30 min after the injection of 10 nmol of calphostin C (41 ± 8% inhibition, n = 5). When mice were pretreated with calphostin C together with the first challenge of morphine, the acute tolerance was completely abolished, whereas there was no significant change with KT-5720 (Fig. 7B). There was no significant change in morphine analgesia with the submaximal dose (0.3 nmol) by pretreatment with calphostin C alone (AUC in pretreatment with vehicle, 1157 ± 333% · min; calphostin C, 1091 ± 200% · min).

As shown in Fig. 8A, Go6976, known as a specific inhibitor of the PKC α and γ isoforms (Wenzel-Seifert et al., 1994) completely reversed the development of morphine tolerance in a dose-dependent manner. Similarly, HBDDE, known as a specific inhibitor of the PKC α and β isoforms (Kashiwada et al., 1994) also completely reversed it in a dose-dependent manner (Fig. 8B). However, less evident reversal of morphine tolerance development was observed with Rottlerin, which is known as a specific inhibitor of the PKC δ isoform (Lu et al., 1997), as shown in Fig. 8C.

Lack of Acute Tolerance of U-69593-Induced Peripheral Analgesia. As in the case of morphine, mice were pretreated with U-69593 at 10 nmol (i.pl.), a dose that is 3 times higher than the maximal dose. The complete recovery of BK responses was also observed 4 h after this pretreatment, as seen in the case with morphine (Fig. 9A). The analgesia by the second challenge with U-69593 (10 nmol) in vehicle-pretreated mice (Fig. 9B) was slightly more potent than in the case with 3 nmol of this compound in naive mice (Fig. 3A). Unlike in the case with morphine, however, there was no significant difference in the U-69593 (10 nmol i.pl.)-induced analgesia between vehicle- and U-69593-pretreated mice (Fig. 9B).

Discussion

The peripheral nociception test used in this study has been developed for the purpose of analyzing in vivo signaling mechanisms at the level of nociceptor endings (Inoue et al., 1998a; Ueda, 1999). This test has several advantages over many other nociception tests. First, it is sensitive enough to assess very weak and short-acting nociceptive responses induced by a local application of small amounts of pain-producing substances (Inoue et al., 1997, 1998a,b). Second, the nociceptive responses in this test are attributed to relatively simple molecular and neuronal mechanisms because a single species of molecule is used for the stimulation of specified receptors on nociceptor endings. Third, because peripheral nociceptors are distant from the dorsal root ganglion containing cell bodies of sensory neurons, the targeting of specific protein expression in nociceptive neurons by AS-ODN (i.t.) techniques is available without effects in peripheral cells.

Here we observed the peripheral morphine analgesia against such BK responses. The local application of morphine into the ipsilateral side of hind paw to the BK-injection side caused a potent inhibition of BK responses (we call it analgesia). However, there was no significant inhibition even when morphine at a 10-times-higher dose was given into the contralateral side or systemically into the back. In addition, the morphine analgesia was blocked by naloxone given into the ipsilateral, but not the contralateral, side. Thus, it is evident that the analgesia by morphine given i.pl. is due to mechanisms on peripheral sites. From the evidence that µ-AS, but not µ-MS pretreatment abolished the morphine-induced peripheral analgesia, it is concluded that the site of morphine action is located on nociceptor endings, as mentioned above.

The type specificity has been often discussed in the opioid
analgesia. As well reported with the central opioid analgesia, the MOR agonists, such as morphine and DAMGO, show more potent analgesia than the DOR or KOR agonists (Hong and Abbott, 1995; Coggeshall et al., 1997). Indeed, U-69593, an agonist with high affinity and specificity to KOR showed 5 or 50 times less potent action in the AD50 values than morphine or DAMGO, respectively. DSLET, a representative DOR agonist, however, showed no significant analgesia (Fig. 3, Table 1). This was supported by the report that DOR function was not observed in the electrophysiological studies with DRG (Abdulla and Smith, 1998), although the gene expression of three types of opioid receptors is observed in the DRG (Hassan et al., 1993; Maekawa et al., 1994; Coggeshall et al., 1997). The lack of DOR function in the peripheral system may be explained by the finding that DORs in the DRG are more located in the Golgi apparatus or in vesicular membranes than in the plasma membranes (Zhang et al., 1998a).

Here we demonstrated that the peripheral morphine analgesia developed acute tolerance in this unique paradigm of experiments. In this study, we took more care in the recovery from the analgesia by the first morphine challenge. As the BK responses when evaluated as percentage of maximal reflex were stable from one mouse to another (see data in Figs. 6–9), the recovery of the BK responses from morphine analgesia could be examined without assessing the control BK response of each mouse before morphine injection. Finally, it was determined that 4 h is required for complete recovery from morphine (or U-69593) analgesia. Under this condition, there was a marked reduction in the morphine analgesia on the second challenge. This finding strongly suggests that the acute tolerance was developed to peripheral morphine analgesia. As higher doses (10 and 30 nmol i.pl.) of morphine still have potent analgesic potencies, the tolerance may be attributed to the desensitization to morphine, but not to the down-regulation, including MOR degradation.

Previously, we reported that DOR is desensitized to repeated challenges of the agonist at a supramaximal concentration in the Xenopus oocytes expressing DOR clone (Ueda et al., 1995). As this desensitization was recovered after the 60-min absence of agonist challenges, it may not be attributed to the down-regulation. Instead, this reduced response was recovered by the treatment with calphostin C, a PKC inhibitor, but not by inhibitors of other protein kinases, such as PKA and calcium-calmodulin kinase II. In that report, the DOR agonist-induced desensitization was not affected by the stimulation of M2-muscarinic receptor, which shares common

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Fig. 6. Development of acute tolerance on morphine-peripheral analgesia. A, time-dependent recovery of BK-induced nociceptive responses after morphine administration. BK (2 pmol)-induced nociceptive activity was represented by the percentage of maximal reflex observed before drug challenges in the beginning of each experiment. The BK response 4 h after the morphine (3 nmol) injection was found to be equivalent to that with vehicle injection. *P < .05, when compared with the vehicle-pretreated mice. B, development of acute tolerance on morphine analgesia. Morphine (3 nmol) was given 4 h after morphine (3 nmol) or vehicle treatment. *P < .05, when compared with the vehicle-pretreated mice at each time point. C, recovery of morphine analgesia by higher doses of morphine. The higher doses of morphine (10–30 nmol) was given 4 h after morphine (3 nmol) or vehicle treatment. *P < .05, when compared with morphine (3 nmol) analgesia in vehicle-pretreated mice. D, no effect of U-69593 analgesia in morphine-treated mice. U-69593 (10 nmol) was given 4 h after morphine (3 nmol) or vehicle treatment. Details are given in the legend to Fig. 1, B and C. All data represent the mean ± S.E. from five to six separate experiments.

Fig. 7. Characterization of acute tolerance development by morphine. A, effects of protein kinase inhibitors on BK responses. Vehicle, calphostin C (Cal C; 10 nmol) or KT-5720 (KT; 10 nmol) was given 4 h before BK treatment. BK-induced nociceptive activity was represented by the percentage of maximal reflex. Details are given in the legend to Fig. 6A. B, recovery of morphine analgesia by calphostin C but not by KT-5720. Vehicle, Cal C, or KT was coadministered with morphine (3 nmol) at 4 h before BK treatment. The results were expressed as analgesia (%), as described in the text. All data represent the mean ± S.E. from six separate experiments. *P < .05, when compared with morphine (3 nmol) analgesia in vehicle and morphine (3 nmol) copretreated mice. Details are given in the legend of Fig. 1B.
signaling including activation of G\(_i1\) and phospholipase C (PLC) with DOR. Thus, PKC activation might be a down-stream mechanism of DOR stimulation, and might desensitize its receptor. Similar desensitizations were also observed with MOR and KOR in *Xenopus* oocytes (Ueda et al., 1996). These findings were also confirmed by other reports using in vivo or culture preparations (Cai et al., 1997; Narita et al., 1997; Kramer and Simon, 1999). Furthermore, such a PKC-mediated desensitization mechanism has been also confirmed by the in vivo experiments in which the analgesic action of DAMGO given i.c.v. was reduced by the i.c.v. pre-
treatment of this opioid peptide; calphostin C attenuated this desensitization (Narita et al., 1997).

The calphostin C-induced reversal of the development of morphine tolerance was confirmed by this study using the peripheral nociception test. One of the advantages of this study is that PKC mechanisms in the acute tolerance could be discussed at the level of well defined nociceptor endings, but not at the level of the central nervous system, consisting of complicated neuronal networks. The involvement of PKC was also confirmed by Go6976 and HBDDE. However, less evident reversal of morphine tolerance development was observed with Rottlerin. Therefore, the PKC isoforms involved in the development of morphine tolerance are characterized to be members of conventional PKC (cPKC) isoforms, which include \(\alpha\), \(\beta\), and \(\gamma\) isoforms, and possess binding sites for phorbol ester, Ca\(^{2+}\), and ATP (Nishizuka, 1992). It is unlikely that this mechanism uses PKC\(d\) isoforms, a member of novel PKC (nPKC) isoform including \(d\), \(e\), \(u\), and \(h\) isoforms, and possessing binding sites for phorbol ester and ATP, but not Ca\(^{2+}\). However, the involvement of atypical PKC (aPKC) isoform remains to be examined because membrane-permeable inhibitors of such isoforms are not available.

Recently, the PKC\(e\) isoform has been reported to be involved in the BK-induced potentiation of neuronal response to painful heat (Cesare et al., 1999). More recently, there has been a report that the PKC\(e\) isoform may be involved in the mechanical, thermal, and chemical hyperalgesia in experiments using PKC\(e\) knockout mice (Khasar et al., 1999). These findings may be consistent with the present results in which BK responses were partially inhibited by calphostin C at 30 min after injection. However, it is unlikely that the inhibitory actions of PKC inhibitors on BK responses affect the BK sensitivity for assessment of morphine analgesia 4 h after calphostin C injection because BK responses themselves were completely recovered at that time (Fig. 7A). As the membrane-permeable inhibitors for the PKCe isoform are not available, the involvement of this isoform in the development of morphine tolerance remains to be determined. It is curious to know whether Rottlerin has an inhibitory action on the PKCe isoform, which is classified into the nPKC family, as well as the PKC\(\delta\) isoform. Regarding the

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**Fig. 8.** Involvement of PKC isoforms in the development of morphine. A to C, recovery of morphine analgesia by Go6976 and HBDDE but not by Rottlerin. Vehicle, Go6976 (0.3 or 3 nmol), HBDDE (3 or 10 nmol), or Rottlerin (3 or 10 nmol) was coadministered with morphine (3 nmol) at 4 h before BK treatment. The results were expressed as analgesia (%), as described in the text. All data represent the mean ± S.E. from four to eight separate experiments. *P < .05, when compared with morphine (3 nmol) analgesia in vehicle- and morphine (3 nmol)-co pretreated mice. The dotted line represents the morphine (3 nmol) analgesia in mice pretreated with vehicle alone, as shown in Fig. 6B.

**Fig. 9.** Lack of tolerance on U-69593-peripheral analgesia. A, time-dependent recovery of BK-induced nociceptive flexor responses after the U-69593 (10 nmol) administration. BK-induced nociceptive activity was represented by the percentage of maximal reflex. Details are given in the legend to Fig. 6A. B, no change of U-69593 analgesia in vehicle- or U-69593-pretreated mice. The results were expressed as analgesia (%), as described in the text. All data represent the mean ± S.E. from six separate experiments. Details are given in the legend of Fig. 1B.
molecular mechanisms of opioid tolerance cAMP hypothesis proposed by Sharma et al. (1995), this hypothesis is now mod-
ifed by the view that opioid mechanism are attenuated by the up-regulation of cAMP and its downstream-signaling molecules during chronic opioid treatments (Nestler et al., 1994). How-
ever, in this study, KT-5720 did not affect the morphine toler-
ance. These findings suggest that PKC mechanisms are in-
volved in the development of acute morphine tolerance, whereas cAMP or its downstream mechanisms, including PKA mechanisms, are not.

In conclusion, we demonstrated that the peripheral MOR-
and KOR-mediated analgesia was observed in a newly de-
veloped BK nociception test. We also demonstrated that the acute tolerance is selectively observed with morphine anal-
gelesis through MOR, and PKC is likely involved in the acute tolerance. As KOR was reported to be easily internalized by KOR agonist treatment whereas MOR is resistant to the internalization by morphine treatment (Sternini et al., 1996; Koch et al., 1998; Zhang et al., 1998b; Li et al., 1999), the present finding showing the selective and acute tolerance of morphine analgesia may be explained by the resistance of MOR to internalization. Histochemical or immune electron microscopical demonstration of opioid receptor internalization corresponding to the acute opioid analgesic tolerance should be an important subject in the future. The present paradigm of peripheral analgesic tests in mice would be useful for the study of in vivo signaling of opioid analgesia.

Acknowledgments

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