**Delta Opioid Receptor Enhancement of Mu Opioid Receptor-Induced Antinociception in Spinal Cord**

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Accepted for publication February 20, 1998 This paper is available online at http://www.jpet.org

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**ABSTRACT**

Although the mu selective agonist [d-Ala²-MePhe⁴-Gly-ol⁶]enkephalin (DAMGO) and the delta selective agonist [d-Pen²,d-Pen⁵]enkephalin (DPDPE) are both antinociceptive when administered directly into the spinal cord of mice, 50% of antinociceptive dose (AD₅₀) of DAMGO is about 2 orders of magnitude lower than the AD₅₀ of DPDPE. In contrast, the two ligands show similar affinities for their respective receptors in vitro binding assays. One possible explanation for this discrepancy is that DPDPE antinociception in the spinal cord is mediated through not delta but mu receptors, for which it has an several hundred-fold lower affinity than DAMGO. In support of this, we found that DPDPE-mediated antinociception was blocked by the mu selective antagonist D-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP). The pA₂ value of CTAP for DPDPE was virtually identical with that for DAMGO. However, because its action also was blocked by naltrindole, an antagonist selective for delta receptors, the latter must also play a role in antinociception. When DAMGO and DPDPE were administered i.t. together at ratios ranging from 1:200 to 1:500, the AD₅₀ of DAMGO was lowered as much as 10-fold relative to its AD₅₀ when given alone. Thus DPDPE had a potentiating effect on DAMGO, although the reverse was not observed. This potentiation was lost in animals made tolerant to systemic morphine. The loss of potentiation seemed to be caused by changes in the delta receptors, because a) the AD₅₀ of DAMGO (i.t.) given alone to tolerant animals was virtually the same as for naive animals, whereas the AD₅₀ of DPDPE given alone increased by 4-fold; and b) the AD₅₀ of DPDPE given alone in the tolerant animal was increased only slightly by naltrindole, whereas CTAP was still a very potent antagonist. We conclude that DPDPE, a selective delta agonist, mediates antinociception in the spinal cord through mu receptors, consistent with results of recent studies of “knock-out” mice lacking mu receptors. At the same time, however, the delta agonist acting through delta receptors can potentiate the mu receptor-mediated antinociceptive action of either mu or delta agonists. This potentiating effect, like the synergistic effect observed between mu receptors at spinal and supraspinal sites, is lost during tolerance.

Morphine administered systemically to humans or laboratory animals induces tolerance. Surprisingly, however, this phenomenon occurs only when the test dose of morphine also is administered systemically. When mice chronically administered morphine by a systemic route are tested by localized injection of morphine either i.e.v. or i.t., no tolerance is observed (Roerig et al., 1984; Roerig and Fujimoto, 1988; He and Lee, 1997).

Insight into this surprising observation has been obtained in studies in which morphine is administered acutely to both supraspinal and spinal sites together. Under these conditions, there is a multiplicative or synergistic effect. The antinociceptive potency at both sites together is significantly greater than the sum of such potencies at individual sites (Yeung and Rudy, 1980; Roerig and Fujimoto, 1989). In tolerant animals, however, the synergism is lost (Roerig et al., 1984). These results suggest that the systemic tolerance emerges from changes not in receptors at discrete central nervous system sites, but rather from changes in interactions between these sites (Roerig and Fujimoto, 1988; He and Lee, 1997).

The opioid receptor types involved in these actions of morphine have not been determined definitively. Both mu and delta receptors are present in both brain and the spinal cord and some studies indicate that both types of receptors may mediate antinociception (Porreca et al., 1987; Miaskowski et al., 1991; Sofuoglu et al., 1991; Suh and Tseng, 1990). However, recent “knock-out” studies indicate that the presence of mu opioid receptors is essential for antinociception of delta as well as mu ligands (Matthes et al., 1996; Sora et al., 1997). In this study we have used the mu and delta receptor-selective agonists DAMGO and DPDPE (James and Goldstein, 1984; Emmerson et al., 1994) and the two antagonists, CTAP and NTI (Portoghese et al., 1988; Gulya et al., 1986; Pelton et al., 1986; Kramer et al., 1989), to delineate the role of the delta

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Received for publication September 4, 1997.

1 This work was supported in part by National Institute on Drug Abuse grants DA-02843 and DA-10048.

ABBREVIATIONS: DAMGO, [d-Ala²-MePhe⁴-Gly-ol⁶]enkephalin; DPDPE, [d-Pen²,d-Pen⁵]enkephalin; CTAP, d-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH₂; NTI, naltrindole; AD₅₀, 50% of antinociceptive dose; i.t., intrathecal; i.e.v., intracerebroventricular.
receptor in antinociception and its relationship with mu receptors in both naive and morphine-tolerant mice.

Materials and Methods

Animals. Male Swiss Webster mice (Hilltop Lab Animals, Inc. Scottsdale, PA) weighing 20 to 25 g were used in this study. They were housed for at least 24 hr before experiments in a temperature- and humidity-controlled environment and fed ad libitum. Each mouse was used only once.

Antinociceptive assay. The antinociceptive assay was a modification of the radiant-heat tail-flick test described by Tulunay and Takemori (1974). The data were made quantal by designating a positive antinociceptive response as one exhibiting an increased latency to tail flick of at least 3 S.D. above the mean latency of the whole group. At least three groups of 10 mice were used to establish dose-response curves and to estimate AD50 values.

Tolerance measurement. Mice were rendered tolerant to morphine by s.c. implantation of one morphine pellet (containing 75 mg morphine free base) for 72 hr. The degree of tolerance was determined as the ratio of the AD50 value of agonist in morphine-pelleted mice to that of morphine-naive mice (Way et al., 1969). The implanted morphine pellet was left intact during the antinociception assay.

Statistics. AD50 values of DAMGO and DPDPE and their 95% confidence limits were calculated by the method of Litchfield and Wilcoxon (1949). The interaction between DAMGO and DPDPE was analyzed in a isobologram. The isobologram was constructed by plotting the AD50 values for DAMGO and DPDPE alone at the i.t. site. The straight line connecting these two points was defined as the theoretical additive line, which consists of points for the purely additive effect at all the ratios between DAMGO and DPDPE. Those values and their S.E. were calculated according to the method of Tallarida et al. (1989). The AD50 values obtained from the experiment were compared with the theoretical additive points and if statistically significant by the Student’s t test, they were indicative of a synergistic effect. If not, the effect was considered additive.

The apparent pA2 values of CTAP and NTI for DAMGO and DPDPE were estimated by the method described by McGilliard and Takemori (1978).

Drugs. Morphine pellets were provided by the National Institute on Drug Abuse (Rockville, MD). DAMGO, DPDPE, NTI, and CTAP were provided by Multiple Peptide Systems (San Diego, CA). The drugs were injected i.t. (Hyden and Wilcox, 1980) in a volume of 5 μl/mouse. DPDPE and NTI were dissolved in 2% 2-hydroxypropyl-β-cyclodextrin (Research Biochemicals, Inc., Natick, MA), and other drugs were dissolved in saline. When the interactive effects between two drugs were tested, they were injected together. All test drugs were administered to mice 30 min before the tail-flick assay, because previous studies had found that morphine induced the maximum effect at this time.

Results

Antinociceptive effect of DAMGO and DPDPE. Intrathecal administration of either DAMGO and DPDPE induced antinociception as determined in the tail-flick assay (fig. 1; table 1). However, the AD50 for DPDPE was about 100-fold higher than that for DAMGO.

Antagonism of DAMGO and DPDPE by NTI and CTAP. When coadministered with different doses of the selective delta receptor antagonist NTI, the effect of DPDPE was decreased significantly, with AD50 values increasing 2- to 4-fold. NTI at doses up to 20 nmol/mouse had no effect on DAMGO antinociception (table 2). Further higher doses were not tested, for some of the animals exhibited unusual behaviors, such as vocalization or scratching at the site of i.t. injection, which suggests possible toxic effects.

The effect of the mu selective antagonist CTAP on DAMGO- and DPDPE-induced antinociception also was tested. As expected, the antinociceptive effect of DAMGO was antagonized significantly by CTAP (table 3). Unexpectedly, however, the same dose range of CTAP also blocked the antinociceptive effect of DPDPE. In fact, a dose of .3 nmol/mouse of CTAP was much more potent than 20 nmol/mouse of NTI in antagonizing the effect of DPDPE. The pA2 values of CTAP and NTI were calculated for DAMGO and DPDPE, respectively. As shown in table 4, the pA2 of CTAP for DAMGO was 11.13, which was virtually identical with that for DPDPE, whereas the pA2 of NTI for DPDPE was 9.0. The pA2 value of NTI for DAMGO could not be determined because the highest concentration of NTI (20 nmol/mouse) used in the study was not able to shift the AD50 of DAMGO significantly (table 4).
TABLE 3
The antagonism effect of CTAP on antinociception induced by DAMGO and DPDPE in the spinal cord

<table>
<thead>
<tr>
<th>CTAP</th>
<th>AD&lt;sub&gt;50&lt;/sub&gt; Values of Peptides (95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPDPE</td>
</tr>
<tr>
<td>nmol/mouse</td>
<td>nmol/mouse</td>
</tr>
<tr>
<td>0</td>
<td>5.8 (3.7–8.9)</td>
</tr>
<tr>
<td>0.01</td>
<td>5.8 (4.3–7.8)</td>
</tr>
<tr>
<td>0.05</td>
<td>18.5</td>
</tr>
<tr>
<td>0.1</td>
<td>(11.2–30.6)</td>
</tr>
<tr>
<td>0.3</td>
<td>38 (24–61)</td>
</tr>
<tr>
<td>0.5</td>
<td>110 (80–151)</td>
</tr>
</tbody>
</table>

Interaction between DAMGO and DPDPE in spinal cord. To analyze the interaction between mu and delta opioid receptors in spinal cord, DAMGO and DPDPE were administered to animals together at different dose ratios, and the data were used to construct an isobologram. A 1:50 or 1:100 ratio of DAMGO/DPDPE was tested first, on the basis of the data were used to construct an isobologram. A 1:50 or 1:100 ratio of DAMGO/DPDPE was tested first, on the basis of an approximately 100-fold difference between the AD<sub>50</sub> values of these agonists. The resulting antinociceptive effect was not significantly different from an additive one by statistical analysis. However, administration of DAMGO and DPDPE at ratios of 1:200 and 1:500 resulted in a significant potentiation (fig. 2, table 5).

Antinociception induced by DAMGO, DPDPE and their combination in the morphine-tolerant animal. Table 1 shows the results of chronic morphine treatment on DAMGO and DPDPE antinociception. Implantation of a single morphine pellet resulted in no tolerance to DAMGO given i.t. However, the same pretreatment resulted in a significant tolerance to DPDPE-induced antinociception, with almost a 4-fold higher AD<sub>50</sub> value. DPDPE-induced antinociception was antagonized in the tolerant animal by both CTAP and NTI (table 6). However, CTAP had a much more potent effect than NTI under these conditions.

When DAMGO and DPDPE were given intrathecally together in morphine-tolerant animals, the potentiating effect of DPDPE on DAMGO antinociception was lost (table 7; fig. 3). Thus the AD<sub>50</sub> of DAMGO when administered in a 1:500 ratio with DPDPE was about half of what it exhibited when administered alone to tolerant animals (table 1), which is indicative of an additive effect.

Discussion

Our results clearly indicate that the delta agonist DPDPE can mediate its antinociceptive effect in the spinal cord through both mu and delta receptors, because both the mu selective antagonist CTAP and the delta selective antagonist NTI can block its antinociception (tables 2–4). We further believe that DPDPE-induced antinociception is mediated directly through mu receptors, but indirectly through delta receptors, by a potentiating effect on the mu receptor. This conclusion is based on the observation that a) the antinociceptive potency of DPDPE in the spinal cord is about 100-fold less than that of DAMGO, although its affinity for delta receptors is about the same as the affinity of DAMGO for mu receptors (Emmerson et al., 1994; James and Goldstein, 1984); and b) DPDPE can potentiate the antinociceptive action of DAMGO on the mu receptor (table 5 and fig. 2).

The relatively low potency of DPDPE to induce antinociception in the spinal cord has been observed previously (So-fuoglu et al., 1991; Malmberg and Yaksh, 1992), and contrasts with binding experiments that indicated the affinity of DPDPE for delta receptors is similar to that of DAMGO for mu receptors (Emmerson et al., 1994; James and Goldstein, 1984). Although these binding experiments were carried out with brain tissue, we have repeated them with use of spinal cord homogenates and observed similar results, i.e., similar affinities of DAMGO for the mu receptor and DPDPE for the delta receptor (unpublished studies). Because each agonist has at least several hundred-fold less affinity for the other receptor, the low antinociceptive potency of DPDPE in the spinal cord is consistent with an antinociceptive action at the mu receptor.

An alternative explanation of the discrepancy between DPDPE binding affinity for the delta receptor and its antinociceptive potency is that DPDPE activation of delta receptors is less efficacious than that of DAMGO on mu receptors. However, more direct evidence for an antinociceptive action of DPDPE on mu receptors is provided by the results of our experiments with the selective antagonists NTI and CTAP. As shown in tables 2 and 3, although the antinociceptive effect of DAMGO, as expected, was blocked by the mu antagonist CTAP but not by the delta antagonist NTI, both antagonists blocked the antinociception induced by DPDPE. Moreover, CTAP was effective against DPDPE at a much lower concentration than NTI.

This conclusion is even clearer when the data in tables 2 and 3 are used to calculate pA<sub>2</sub> values (table 4). The pA<sub>2</sub> value of CTAP for DAMGO was virtually identical with that for DPDPE (about 11 in each case), which strongly indicates that DAMGO and DPDPE act on the same site, presumably the mu receptor. The pA<sub>2</sub> value of NTI for DPDPE was about 9.0, whereas the pA<sub>2</sub> value of NTI for DAMGO could not be calculated because up to 20 nmol/mouse of NTI was not able to increase the AD<sub>50</sub> of DAMGO even 2-fold. Thus DPDPE binds to a second receptor, presumably delta, with which DAMGO does not interact. Although the slopes for CTAP antagonism of both DAMGO and DPDPE were close to unity, the slope for NTI antagonism of DPDPE was −3.1. This suggests that DPDPE-mediated antinociception involves a different kind of interaction at the delta receptor from that at mu receptors.

TABLE 4
Determination of the apparent pA<sub>2</sub> values of CTAP and NTI in antagonism of DAMGO and DPDPE in morphine-naive mice

<table>
<thead>
<tr>
<th>Antagonists</th>
<th>DAMGO</th>
<th>DPDPE</th>
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<tbody>
<tr>
<td></td>
<td>pA&lt;sub&gt;2&lt;/sub&gt; Values (95% confidence limits)</td>
<td>Slope ± S.E.</td>
</tr>
<tr>
<td>CTAP</td>
<td>11.13 (10.15–12.11)</td>
<td>−1.46 ± 0.31</td>
</tr>
<tr>
<td>NTI</td>
<td>N.D.*</td>
<td>N.D.*</td>
</tr>
</tbody>
</table>

* The pA<sub>2</sub> value of NTI for DAMGO and its slope could not be determined because NTI, up to 20 nmol/mouse, was not able to increase the AD<sub>50</sub> of DAMGO even 2-fold.
the mu receptor, which we believe is further evidence for an indirect, potentiating effect at the delta receptor.

More direct evidence for DPDPE-mediated potentiation of agonist at the mu receptor was obtained when we examined the interaction between DAMGO and DPDPE when given simultaneously to animals. Previous studies by other groups have reported synergistic effects between opioid ligands in general (Horan et al., 1992; Porreca et al., 1987; Roerig et al., 1991; Rossi et al., 1993, 1994), and between DAMGO and DPDPE in particular (Malmberg and Yaksh, 1992; Roerig and Fujimoto, 1989).

Different results were obtained depending on the DAMGO/DPDPE ratio used (table 5). Ratios of 1:50 and 1:100 produced only additive effects. However, at the higher DPDPE ratios of 1:200 and 1:500, potentiation of the DAMGO effect was observed. Under the latter conditions, the AD$_{50}$ for DAMGO was reduced to about 10% of that exhibited when DAMGO was administered alone. The AD$_{50}$ for DPDPE was reduced only slightly, which indicates that there was no

<table>
<thead>
<tr>
<th>Ratios</th>
<th>AD$_{50}$ Values of Peptides (95% confidence limits)</th>
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<tbody>
<tr>
<td></td>
<td>DAMGO</td>
</tr>
<tr>
<td></td>
<td>nmol/mouse</td>
</tr>
<tr>
<td>1:50</td>
<td>0.026 (0.01–0.04)</td>
</tr>
<tr>
<td>1:100</td>
<td>0.02 (0.01–0.035)</td>
</tr>
<tr>
<td>1:200</td>
<td>0.011 (0.006–0.024)</td>
</tr>
<tr>
<td>1:500</td>
<td>0.005 (0.003–0.008)</td>
</tr>
</tbody>
</table>

The statistical significance was obtained by the $t$ test (* $P < .05$), which indicates a synergistic effect.

The results in table 5 indicate that delta receptor-mediated potentiation of mu receptor-mediated antinociception, however, is observed only at relatively high concentrations of DPDPE, much higher than its affinity for delta sites as determined by in vitro binding assays. Therefore, it seems that the efficacy of DPDPE acting at these sites is very low,
perhaps because of poor coupling to a G-protein. Further studies will be necessary to address this question and to shed light on the mechanism by which delta receptor activation potentiates mu receptors. In particular, it is not clear whether coupling occurs through physical association of mu and delta receptors, or is mediated by a sequence of reactions.

In morphine-tolerant animals, DPDPDE potentiation of DAMGO is lost. As shown in figure 3 and table 7, the two ligands at all ratios tested now produced only an additive effect. This loss of potentiation apparently is caused by changes in the delta receptors. As shown in table 1, the AD50 of DPDPDE was increased 4-fold in morphine-tolerant animals (table 1), in agreement with another study (Porreca et al., 1987). This increase is unlikely to reflect reduced affinity for mu receptors, for there was no change in the AD50 of DAMGO in the morphine-pelleted animals (table 1). We and others also have shown previously that the AD50 of i.t. morphine also was unchanged in these systemically tolerant animals (Roerig, et al., 1984; He and Lee, 1997), although Porreca et al. (1987), by use of an acute tolerance paradigm, reported an increase in AD50 of i.t. morphine. Thus we conclude that in morphine-pelleted animals changes in spinal cord delta receptors occur that reduce its ability to potentiate mu receptor-mediated antinociception.

This conclusion is supported further by studies with selective antagonists in tolerant animals. The delta antagonist NTI had a relatively modest effect on DPDPDE-induced antinociception in morphine-tolerant mice. A dose of 10 nmol/mouse given i.t. increased the AD50 of i.t. DPDPDE from 21 nmol/mouse in naive animals to 52 nmol/mouse in tolerant animals (table 6). In contrast, i.t. administration of the mu selective antagonist CTAP (0.05 nmol/mouse) raised the AD50 of DPDPDE from 18.5 nmol/naive mouse to 232 nmol/tolerant mouse (table 6). We interpret these results to mean that delta receptor interaction contributes little to the antinociceptive effect of the delta agonist in tolerant animals.

Other laboratories have demonstrated previously that synergistic interactions occur between mu receptors at spinal and supraspinal levels (Yeung and Rudy, 1980), and that these interactions, like the potentiation demonstrated here, are lost during morphine tolerance (Roerig et al., 1984). Recently, we demonstrated that dynorphinA-(2–17), which does not interact directly with opioid receptors but which is capable, like full-length dynorphin, of modulating some of the acute and chronic effects of morphine (Friedman et al., 1981; Takemori et al., 1993), was capable of partially restoring spinal-supraspinal morphine synergism in morphine-tolerant animals (He and Lee, 1997). However, dynorphinA-(2–17) has no effect on DPDPDE potentiation of DAMGO antinociception at the i.t. site in morphine-tolerant animals (unpublished data).

In conclusion, DPDPDE apparently has two distinct actions in the spinal cord. It mediates antinociception by interacting with mu receptors, consistent with its sensitivity to CTAP (table 3), and it potentiates mu receptor-mediated antinociception by acting through delta receptors, consistent with antagonism by NTI (table 2). Thus, although DAMGO and DPDPDE both mediate antinociception through mu receptors, DPDPDE apparently potentiates its relatively weak affinity for these receptors through simultaneous action at the delta receptor.

The conclusion that DPDPDE mediates antinociception through mu rather than delta receptors seems to be inconsistent with other studies reporting delta receptor-mediated antinociception. However, it is important to emphasize that this conclusion is based largely on studies showing that delta selective antagonists block delta agonist-mediated antinociception (Mattia et al., 1992; Crook, et al., 1992; Tseng, et al., 1997). As we have shown here, this observation can be explained by an indirect, potentiating effect of delta receptor on mu receptor, without the necessity of concluding that delta receptors directly mediate antinociception. Although some studies have reported that mu antagonists were ineffective in blocking delta antinociception (Qi et al., 1990; Tseng, et al., 1997), at least one other study with CTAP agreed with our results that this mu antagonist could block delta antinociception (Kramer et al., 1989). This discrepancy may reflect the fact that different delta agonists and mu antagonists have different affinities for their selective receptors as well as for the opposite receptor. In any case, our conclusion of delta agonist-mediated antinociception through the mu receptor is also consistent with recent studies of mu receptor knock-out animals, which indicate that these receptors are critical for antinociception of delta as well as mu agonists (Matthes et al., 1996; Sora et al., 1997).

We have evidence for a similar potentiating effect in the brain, one which is also lost during chronic systemic morphine treatment (unpublished data). Because the AD50 of i.t. morphine is not changed in animals tolerant to systemic morphine (He and Lee, 1997), this potentiation may serve to regulate the activity of endogenous delta ligands in vivo. In fact, the existence of delta receptor potentiation of mu receptor activation suggests the possible need for reinterpretation of the receptor selectivity of some ligands. For example, the apparent high affinity of some agonists, such as β-endorphin, for both mu and delta receptors may reflect delta potentiation of mu interaction. Detailed studies of in vitro binding in the presence of selective antagonists could help to resolve this issue.

Acknowledgments

The authors thank Drs. E. L. Way and Andy Smith for valuable suggestions.

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