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Supraspinal Delta$_2$ Opioid Agonist Analgesia in Swiss-Webster Mice Involves Spinal GABA$_A$ Receptors

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RADY, J. J. AND J. M. FUJIMOTO. *Supraspinal delta$_2$ opioid agonist analgesia in Swiss-Webster mice involves spinal GABA$_A$ receptors.* PHARMACOL BIOCHEM BEHAV **54**(2) 363-369, 1996. – The tail-flick response is a spinal reflex that can be modulated by administration of antinociceptive agents supraspinally through activation of descending systems and involvement of the action of neurotransmitters in the spinal cord. Descending noradrenergic and serotonergic systems are involved in morphine (and other μ opioid receptor agonists)-induced antinociception. These descending systems, however, are not involved in supraspinal δ opioid receptor agonist-induced antinociception. Recently, a descending system mediated by spinal gamma-aminobutyric acid (GABA) A and B receptors has been demonstrated to be involved in the antinociceptive action of δ_1 opioid receptor agonists ([D-Pen^{2,5}]enkephalin in ICR mice and [D-Pen^{2,5}]enkephalin and heroin in Swiss-Webster mice). In the present study, the involvement of spinal GABA$_A$ receptors in the antinociceptive action of supraspinal δ_2 opioid receptor agonists, [D-Ser^2]-Leu-enkephalin-Thr and 6-monoacetylmorphine, action was demonstrated. The intrathecal administration of GABA$_A$ receptor antagonists, bicuculline and picrotoxin, inhibited the antinociceptive action of both [D-Ser^2]-Leu-enkephalin-Thr and 6-monoacetylmorphine given intracerebroventricularly. The intrathecal administration of 2-hydroxysaclofen, a GABA$_B$ receptor antagonist, had no effect. These studies suggest that supraspinal δ_2, like δ_1, opioid receptor action involves spinal GABA$_A$ receptors, but δ_2, unlike δ_1, action does not involve GABA$_B$ receptors. Thus, the supraspinal δ_1 agonist action (heroin, DPDPE) and the δ_2 agonist action (6MAM, DSLET) can be further differentiated by the selectivity of the spinal GABA receptors involved in Swiss-Webster mice.

6-Monoacetylmorphine DSLET Delta$_2$ opioid receptor GABA$_A$ GABA$_B$ Antinociception Descending pathway

IN studying the mechanism of action of heroin, its antinociceptive action has served as a convenient measure of one of its pharmacological actions (26,27,31-34). Recently, we assessed the opioid receptor selectivity of heroin (3,6-acetylmorphine) and 6-monoacetylmorphine (MAM) using the tail-flick test in mice (17,19,20). In this test, the tail-flick response to noxious heat is primarily a spinal reflex, as evidenced by the response remaining intact after transection of the spinal cord (1,4,30). Opioids can inhibit the response by direct action (after intrathecal, IT, administration) within the spinal cord (1,4,36, 37,39,40) or indirect action (after intracerebroventricular, ICV, administration). In the latter case, activation of descending neuronal systems inhibit the tail-flick response (8,38). Descending pathways (as elucidated most widely in rats) involve mediation by norepinephrine and serotonin (8,37,38). A convenient way in which to demonstrate descending norepinephrine and serotonin pathways in mice is to administer selective antagonists (for example, yohimbine and methysergide, respectively) to inhibit the antinociception resulting from ICV administration of opioids (8,35). Administration of methysergide and yohimbine IT inhibits the antinociception produced by the μ agonist action of ICV heroin, morphine and Tyr-D-Ala^2-N-MePhe^4-Gly-ol^5 (DAMGO, a peptide that is selective for the μ opioid receptor) (3,19,24,35), while antinociception produced by κ opioid agonists given ICV is attenuated by IT methysergide (17,29). The serotonergic and noradrenergic systems are not involved in ICV δ opioid receptor agonist-induced antinociception because neither methysergide nor yo-

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himbine given IT are effective inhibitors (17). The descending pathway involved in the antinociceptive action of δ receptor agonists takes on added interest because in Swiss-Webster mice heroin and MAM act in the brain on δ rather than μ receptors, even though μ receptors (on which morphine continues to act) are present (17,19). Note that sequential removal of the three and six acetyl groups from heroin yields MAM and morphine.

The antinociception induced by [D-Pen^{2,5}]enkephalin [DPDPE, a δ_1 opioid receptor selective peptide agonist (13,20–22,25)] administered ICV in ICR and Swiss-Webster mice involves spinal $GABA_A$ and $GABA_B$ receptors (11,18). Administration of bicuculline, picrotoxin, and 2-hydroxysaclofen IT inhibits DPDPE-induced antinociception. Likewise, in the special case where ICV heroin acts on δ_1 receptors in Swiss-Webster mice, spinal $GABA_A$ and $GABA_B$ receptors are involved (18–20).

In the present investigation, studies were designed to determine whether the antinociceptive action produced by the activation of δ_2 receptors by MAM in the brain of Swiss-Webster mice involved both GABA receptors. Even though heroin acts on δ_1 receptors, MAM acts on δ_2 receptors in brain of Swiss-Webster mice (17,20). [D-Ser^2]-Leu-enkephalin-Thr (DSLET) was used as a standard for δ_2 agonist action. We chose DSLET because Portoghese, Takemori and colleagues (25) used DSLET as the δ_2 agonist in evaluating the δ_2 receptor antagonist property of naltriben. We subsequently used naltriben to determine that MAM acted on δ_2 receptors in the brain of Swiss-Webster mice (20). $GABA_A$ receptor involvement was assessed by administration of bicuculline and picrotoxin IT and $GABA_B$ involvement was assessed by administration of 2-hydroxysaclofen IT. The results demonstrate that activation of supraspinal δ_2 receptors produces antinociception that involves $GABA_A$, but not $GABA_B$, receptors in the spinal cord. The results add to the ability to discriminate not only between the δ_1 receptor action of heroin and δ_2 action of MAM but also the associated spinal GABA receptors involved.

METHOD

Animals and Measurement of Antinociception

Male Swiss-Webster mice weighing 25–40 g (Hilltop Lab Animals, Scottdale, PA) were used for all studies. Each mouse was only used once. Antinociception was measured by the radiant heat tail-flick test (6). The control latencies were 2–4 s, and a 10 s cutoff time was used to prevent injury to the tail. The post treatment latencies in s were converted to percent maximum possible effect (% MPE) according to the method of Dewey et al. (7):

$$\% \text{ MPE} = \frac{100\,(\text{postdrug time} - \text{predrug time})}{(10 - \text{predrug time}).}$$

The mean % MPE and standard error of the mean (SEM) for each treatment group (consisting of 8 to 10 mice) were calculated to make comparisons between treatments.

Statistical Analysis

For the antagonist dose response data, the mean % MPEs for the treatment groups were compared to one control group using Dunnett's test. Student's *t*-test was used for comparison of the treatment group to the matched control group in the studies on the duration of action of the antagonists. Differences were considered significant at a $p \leq 0.05$ (23). Agonist dose response data were fitted to straight lines and the slopes and ED_{50} values were determined and compared using the method of Litchfield and Wilcoxon as described by Dewey et al. (7).

Drug Sources and Administration

Picrotoxin and (+)-bicuculline were obtained from Sigma (St. Louis, MO). DSLET was purchased from Peninsula Laboratories (Belmont, CA). 2-Hydroxysaclofen was obtained from Research Biochemicals International (Natick, MA). MAM (free base) was obtained from the National Institute on Drug Abuse (Rockville, MD). All doses of drugs stated hereafter refer to the form as stated here. DSLET was dissolved in a 0.1% Triton X-100 solution in 0.9% saline. All other compounds were dissolved in 0.9% saline solution. Slight heating was needed to aid in dissolving 2-hydroxysaclofen. To aid in dissolving the bicuculline and MAM a few drops of 0.1 M hydrochloric acid was added and the solution was heated slightly.

The opioid agonists were administered ICV in a volume of 4 μl (10), under light halothane anesthesia. The GABA antagonists and saline were given IT in a 5 μl volume (12).

Protocols

Administration of various doses of the antagonists IT while keeping the time of antagonist administration and dose and time of agonist administration constant allowed for determination of the dose-response relationships for the antagonists. The duration of action for the antagonists was determined by varying the time of antagonist administration while the other parameters remained constant. The overall effect of the antagonists on the antinociceptive action of the δ_2 opioid receptor agonists was determined by using a number of doses of the agonist given ICV at 10 min while saline or an antagonist (at a fixed dose) was administered IT at 5 min before the tail-flick test. All experiments were performed in compliance with the Institutional Animal Care and Use Committee (Animal Studies Subcommittee).

RESULTS

Dose-Response Relationships for the Antagonists Given IT Against ICV DSLET-Induced Antinociception (Fig. 1)

Bicuculline administered IT (5 min before the tail-flick test) dose dependently antagonized the antinociceptive action of DSLET (0.4 μg) given ICV (Fig. 1A). Doses of 0.5 μg and 1 μg of bicuculline produced a significant decrease in the antinociceptive activity of DSLET. For the remainder of the studies with bicuculline the 0.5 μg dose was used. The antinociceptive action of ICV DSLET was also reduced by administration of IT picrotoxin at doses of 0.25 and 0.5 μg (Fig. 1B). The 0.25 μg dose of picrotoxin was used for the subsequent studies. These results suggested that the antinociceptive action of ICV DSLET involved $GABA_A$ receptor action in the spinal cord.

Unlike the antagonistic action observed for the $GABA_A$ antagonists, administration of 2-hydroxysaclofen (the $GABA_B$ receptor antagonist) had no effect on ICV DSLET-induced antinociception (Fig. 1C). The 7.5 μg dose has been shown previously to inhibit IT $GABA_B$ agonist (baclofen)-induced as well as ICV δ_1 agonist (DPDPE, heroin)-induced antinociception (11,18). Increasing the dose of 2-hydroxysaclofen resulted in severe behavioral effects such as hindlimb flacidity and paralysis in the mice. The 7.5 μg dose was used for the remainder of the studies. It did not affect the antinociceptive action of IT isoguvacine, a $GABA_A$ agonist. Nor did IT picrotoxin

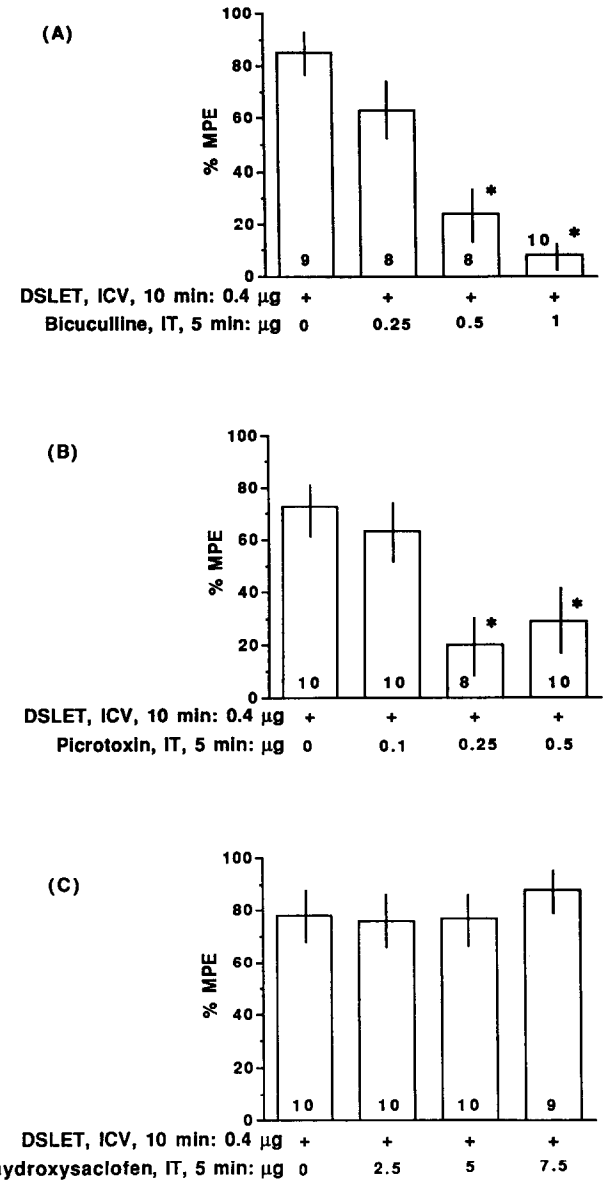

FIG. 1. Dose–response relationships for the effect of GABA antagonists, given IT, against ICV DSLET-induced antinociception. (A–C) DSLET, given ICV at a dose of 0.4 μg 10 min before the tail flick test, produced a high level of antinociception (group 1). (A) This antinociception was reduced by administration of bicuculline IT at doses of 0.5 μg and 1 μg (group 1 vs. 3 and 4). *Significantly different from the control group (given DSLET only) using Dunnett's test; $p \leq 0.05$. (B) Picrotoxin, given IT, at doses of 0.25 μg and 0.5 μg decreased the antinociceptive action of ICV DSLET (group 1 vs. group 3 and 4). *Significantly different from the control group (given DSLET only) using Dunnett's test; $p \leq 0.05$. (C) DSLET-induced antinociception remained high following 2-hydroxysaclofen administration IT. No significant differences were found using Dunnett's test. In this figure and the next one, the bar represents the mean % MPE for each group and the vertical line at the top of the bar indicates SEM. The number at the bottom of the bar indicates the number of mice used for each group. The groups are numbered 1 . . . *n* starting with the left-most group. In this figure a + under the bar indicates that DSLET was administered as stated to the left and the number given under the bar indicates the dose for antagonist administration; a 0 means that saline was administered instead of the antagonist.

and bicuculline inhibit the action of IT baclofen, a GABA$_B$ agonist (data not shown). The present results suggested that ICV δ_2 agonist-induced antinociception did not involve spinal GABA$_B$ receptor activation.

Duration of Action of IT GABA Antagonists on ICV DSLET-Induced Antinociception (Fig. 2)

Bicuculline (0.5 μg) given IT antagonized DSLET antinociception when administered from 5 min up to 30 min before the tail flick-test (Fig. 2A). The antagonism of DSLET antinociception was no longer present at 45 min. The duration of action for IT picrotoxin (0.25 μg) antagonism of ICV DSLET-induced antinociception (Fig. 2B) was similar to that for bicuculline. Picrotoxin administration from 5 min to 30 min before the tail-flick test produced antagonism of the antinociceptive action of ICV DSLET. The antagonism of DSLET antinociception was no longer obtained at 45 min. The finding that both IT bicuculline and picrotoxin antagonize ICV DSLET-induced antinociception indicated that a descending neuronal pathway mediated by spinal GABA$_A$ receptors was involved when δ_2 receptors were activated in the brain.

The lack of 2-hydroxysaclofen effect above could be due to the time of administration, even though the 5-min time point was used in the previous studies (11,18). Therefore, the 7.5 μg dose of 2-hydroxysaclofen was administered IT at various time points along with ICV DSLET at 10 min (Fig. 2C). When 2-hydroxysaclofen was administered up to 30 min before the tail-flick test, no effect on DSLET antinociception was seen. This result suggested that the time of administration was probably not a problem. This study, like the dose-response experiment for 2-hydroxysaclofen, suggested that spinal GABA$_B$ receptors were not involved in supraspinal DSLET analgesia. The administration time of 5 min before the tail-flick test was chosen for all of the antagonists for the remainder of the studies.

The Effect of GABA Antagonists Given IT on the Dose-Response Relationships for ICV DSLET (Fig. 3)

DSLET administered ICV (10 min before the tail-flick test) produced dose-dependent antinociception. In these mice, saline was given IT 5 min before the tail-flick test (closed circles). The ED$_{50}$ value (95% confidence interval) for ICV DSLET was 0.12 (0.06 to 0.24) μg Administration of bicuculline IT 5 min before the tail-flick test produced a sixfold rightward parallel shift of the DSLET dose-response curve (open circles). The ED$_{50}$ value for ICV DSLET in the presence of IT bicuculline was 0.67 (0.49 to 0.98) μg. Similarly, IT administration of picrotoxin 5 min before the tail-flick test produced a ninefold parallel rightward shift of the DSLET dose-response curve [shifting the ED$_{50}$ value to 1.03 (0.55 to 1.94) μg; open squares]. The ED$_{50}$ value for ICV DSLET was not altered significantly by the IT administration of 7.5 μg of 2-hydroxysaclofen [the ED$_{50}$ value was 0.18 (0.10 to 0.34) μg; open triangles]. Thus, these studies reinforced the evidence that spinal GABA$_A$, not GABA$_B$, receptors were involved in ICV DSLET-induced antinociception. Because DSLET is a prototypic δ_2 receptor agonist that is a peptide, the next series of experiments involved an alkaloid that has δ_2 receptor agonist activity (17,20).

The Effect of GABA Antagonists Given IT on the Dose-Response Relationship for MAM Given ICV (Fig. 4)

ICV administration of MAM 10 min before the tail-flick test produced dose-dependent antinociceptive responses (closed

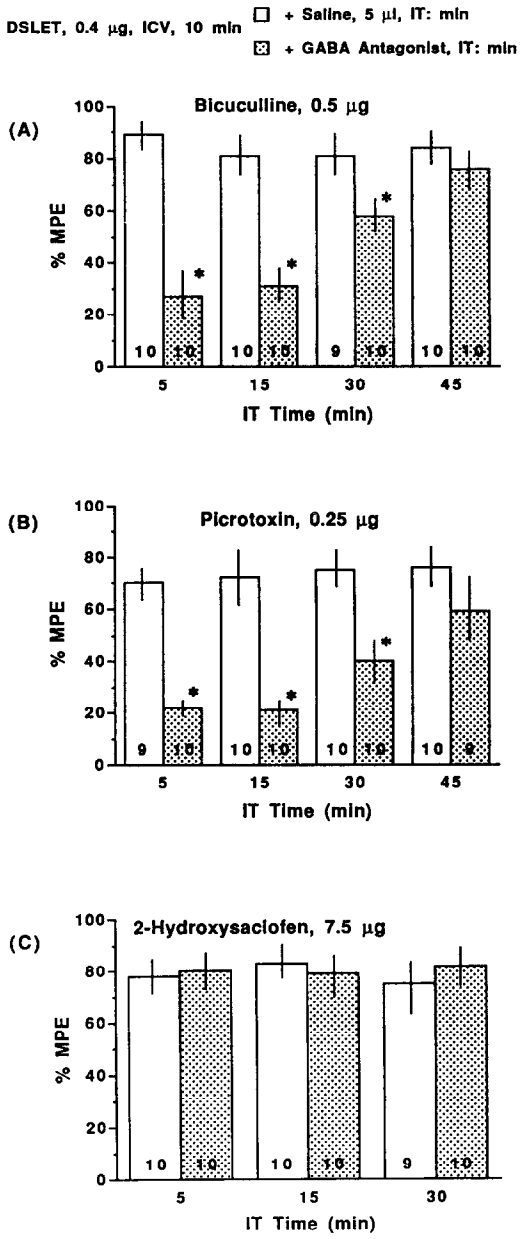

FIG. 2. Duration of action for the effect of the GABA antagonists given IT against ICV DSLET-induced antinociception. (A-C) Administration of DSLET (0.4 μg) ICV 10 min before the tail flick test produced a highly antinociceptive response no matter which time point was used for IT saline administration (open bars). (A) Bicuculline (0.5 μg) administered IT (hatched bars) reduced ICV-DSLET-induced antinociception when administered from 5 min to 30 min before the tail flick test. At 45 min before the tail flick test bicuculline administration no longer antagonized DSLET antinociception. *Significantly different from the time matched saline control group using Student's t-test; $p \leq 0.05$. (B) Administration of IT picrotoxin (0.25 μg; hatched bars) decreased the antinociceptive action of ICV DSLET when given from 5 min to 30 min before the tail flick test. This antagonistic effect of picrotoxin against DSLET antinociception was no longer present at the 45 min time point. *Significantly different from the time matched saline control group using Student's t-test; $p \leq 0.05$. (C) Administration of 2-hydroxysaclofen (7.5 μg) IT (hatched bars) did not alter DSLET-induced antinociception at any of the time points used (5 min to 30 min before the tail flick test). No significant differences were found using Student's t-test. For this figure, the number under the bar indicates the time before the tail flick test for IT administration of saline or the antagonist and the number inside the bar indicates the number of mice used for each group. All other designations are as in Fig. 1.

circles). Saline was given IT 5 min before the tail-flick test in these mice. The dose-response curve for ICV MAM was shifted to the right in a parallel fashion by administration of bicuculline IT at 5 min (open circles). The ED_{50} value changed from 2.6 (1.2 to 5.8) μg to 16.1 (11.3 to 22.9) μg, which represented a sixfold parallel shift. A sevenfold rightward parallel shift in the dose-response curve of ICV MAM was observed when picrotoxin was administered IT (open squares); the ED_{50} value for MAM in the presence of picrotoxin was 18.5 (13.4 to 25.5) μg. Therefore, like ICV DSLET, ICV MAM produced antinociception that involved activation of spinal $GABA_A$ receptors.

Also like DSLET, the antinociceptive activity of MAM was not altered by administration of 2-hydroxysaclofen, 7.5 μg, IT (open triangles). The ED_{50} value for ICV MAM in the presence of IT 2-hydroxysaclofen was 6.5 (3.1 to 13.8) μg, which was not significantly different from the ED_{50} value of the MAM when saline was given IT. Thus, supraspinal MAM-induced antinociception did not involve spinal $GABA_B$ receptor activation. Thus, δ_2 receptor activation in the brain by DSLET and MAM produced antinociception mediated by spinal $GABA_A$, but not $GABA_B$, receptors.

DISCUSSION

In the present study the δ_2 receptor agonists (DSLET and MAM) given ICV in Swiss-Webster mice, produced antinociception that was attenuated by IT administration of bicuculline and picrotoxin, but not 2-hydroxysaclofen. These results indicated that δ_2 receptor activation in the brain produced antinociception that involved $GABA_A$, but not $GABA_B$, receptors in the spinal cord. Previous publications show that the antinociception induced by δ_1 opioid receptor agonists given ICV is mediated by spinal $GABA_A$ and $GABA_B$ receptors in both ICR and Swiss-Webster mice (11,18). Further discussion is necessary on certain aspects of the interpretation of the present results.

An argument based on lack of effect of an antagonist requires cautious interpretation. Only one dose of 2-hydroxysaclofen was used IT, and it did not inhibit either ICV DSLET- or MAM-induced antinociception. Increasing the dose of 2-hydroxysaclofen above 7.5 μg resulted in hind limb flacidity and in a few cases paralysis; therefore, higher doses of 2-hydroxysaclofen could not be used. The 7.5 μg dose of 2-hydroxysaclofen was shown previously to inhibit IT baclofen (a $GABA_B$ receptor agonist)- and ICV DPDPE-induced antinociception in both ICR and Swiss-Webster mice (11,18). Furthermore, the analgesia produced by cold-water swim stress involves a supraspinal δ_2 receptor response (28), which in ICR mice is inhibited by IT administration of bicuculline and picrotoxin (14) but not by the 7.5 μg of 2-hydroxysaclofen given IT (unpublished data). Therefore, the antinociception produced by supraspinal δ_2 receptor activation (by means other than ICV DSLET and MAM) also appears to involve only spinal $GABA_A$ receptors.

Even though the present results may be interpreted as showing a complete separation between the $GABA_A$ receptor involvement for δ_2 agonist action and $GABA_A$ and $GABA_B$

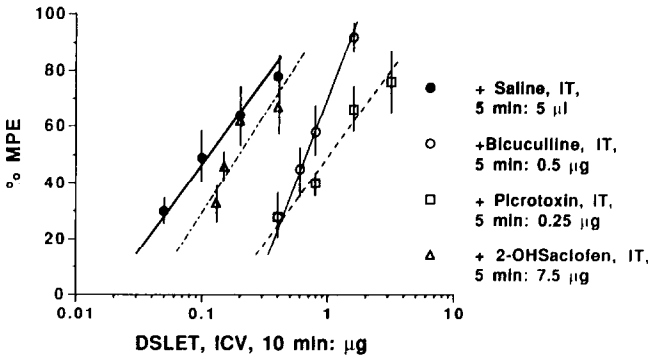

FIG. 3. Dose-response curves for ICV DSLET-induced antinociception with saline or the GABA antagonists given IT. DSLET given ICV (10 min before the tail flick test) with saline given IT (5 μl, 5 min before the tail flick test) produced a dose-dependent antinociceptive response (closed circles). The dose-response curve for DSLET antinociception was shifted to the right sixfold in a parallel fashion by the administration of 0.5 μg of bicuculline IT 5 min before the tail flick test (open circles). Administration of IT picrotoxin (0.25 μg, 5 min before the tail flick test) also produced a parallel, rightward shift of the DSLET dose response curve, approximately ninefold (open squares). The DSLET dose-response curve was not altered by administration of the 7.5 μg dose of 2-hydroxysaclofen (abbreviated 2-OHSaclofen) 5 min before the tail flick test (open triangles). For this figure and the next one, each point represents the mean % MPE for groups of 8 to 10 mice given each dose of agonist. The vertical line indicates the SEM.

involvement in δ_1 agonist action, note that a small rightward (though not statistically significant) shift in the MAM and DSLET dose-response curves seemed to occur in the presence of 2-hydroxysaclofen. Thus, it was possible that a small contribution from GABA$_B$ receptors did occur. In the situation where both GABA$_A$ and GABA$_B$ receptors are involved (as with ICV DPDPE), a restricted condition can be created to show that the combination of 2-hydroxysaclofen with either picrotoxin or bicuculline is more effective than with each of the antagonists alone (11). Such a superadditive effect was not obtained in attempting to inhibit ICV MAM-induced antinociception in the present study (results not given). Thus, the evidence presented diminishes but does not eliminate the caveat of the lack of effect of 2-hydroxysaclofen.

It must be noted that DSLET interacts with μ receptors, although with a much lower affinity than that for δ receptors (5,9). Also, MAM is primarily a μ agonist in ICR mice (17), even though it is a δ_2 agonist in Swiss-Webster mice (17). It is unlikely that the μ receptor action of DSLET and perhaps MAM involves spinal GABA receptor mediation because ICV morphine (which is commonly used as a μ receptor agonist) produces antinociception in Swiss-Webster mice, which is not altered by administration of the GABA antagonists (18). In contrast to the latter finding in mice, there is a suggestiion that in the rat, there is a descending serotonergic antinociceptive system (similar to that activated by μ agonists) that may involve GABA at the spinal level (2). In the present study, the slopes of the dose-response curves for MAM and DSLET in the presence of bicuculline and picrotoxin appeared in some cases to be steeper than the control curves (though not statistically significant). A steeper slope would suggest that the effect from lower agonist doses was inhibited better by the antagonists than that from higher doses. At higher agonist doses, μ receptors may be activated because of increased concentrations leading to wider spread to sites beyond those reached by the lower doses.

The reason for the difference between ICV δ_2 (involving only GABA$_A$ receptors) and δ_1 agonist action (GABA$_A$ and GABA$_B$ receptors) requires further discussion. It is possible that two separate descending pathways exist, one mediated by spinal GABA$_A$ receptors and the other by GABA$_B$ receptors, and that these δ receptor subtypes are located in different sites in the brain. In the rat, microinjection of L-glutamate into medial sites in the medulla (such as the nucleus raphe magnus) produces antinociception mediated by GABA$_B$ receptors in the spinal cord, whereas that produced by L-glutamate at lateral sites (such as the nucleus gigantocellularis pars alpha) is mediated by GABA$_A$ receptors (15,16). Thus, the differential activation of spinal GABA receptors observed for supraspinal δ_1 and δ_2 antinociception could be due to δ_1 agonists acting at both medial and lateral sites, while δ_2 agonists only act at the lateral sites. However, it is not known whether the L-glutamate/GABA systems in the rat are the same as the δ receptor/GABA systems in mice. In the present study, it is unlikely that selective distribution to different sites in the brain after ICV administration or differences between an alkaloid and a peptide were involved. Each pair, DSLET and MAM for δ_2, and DPDPE and heroin for δ_1, included a peptide and an alkaloid; δ receptor selectivity and associated GABA receptor involvement were not dependent on the chemical class.

In summary, the antinociceptive activity of δ_2 receptor agonists was reduced by administration of GABA$_A$ antagonists given IT. This effect is similar to that observed for δ_1 receptor activity. However, unlike δ_1 receptor action, δ_2 receptor-induced antinociception was not decreased by IT GABA$_B$ antagonist administration. While supraspinal δ_1 receptor agonist action involves activation of both GABA$_A$ and GABA$_B$ receptors in the spinal cord, supraspinal δ_2 receptor antinociception involves activation of only the GABA$_A$ receptors in the spinal

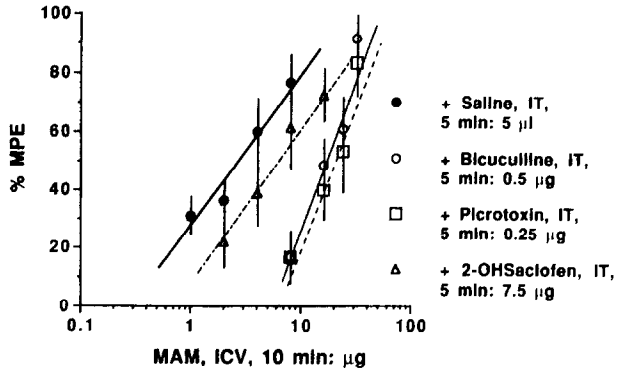

FIG. 4. Dose-response curves for ICV 6MAM in the presence of saline or the GABA antagonists given IT. Administration of ICV 6MAM (10 min before the tail flick test) with saline given IT produced dose-dependent antinociception (closed circles). The dose-response curve for 6MAM antinociception was shifted to the right, approximately sixfold, in a parallel manner by administration of IT bicuculline (open circles). Administration of picrotoxin IT produced a similar shift (about sevenfold) of the 6MAM dose-response curve (open squares). The dose response curve for ICV 6MAM-induced antinociception was not affected by 2-hydroxysaclofen (abbreviated 2-OHSaclofen) administration (open triangles).

cord. The ability to selectively inhibit δ_1 and δ_2 receptor actions of heroin and MAM, respectively, and the corresponding spinal GABA receptor-mediated responses provides a means for assessing the importance of the biotransformation of heroin to MAM and morphine in Swiss-Webster mice. Preliminary results based on the administration of a δ receptor antagonist indicate that the biotransformation of heroin and MAM to morphine appears not to occur as rapidly as expected (17).

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