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Ethylketocyclazocine and Bremazocine Analgesia in Neonatal Rats

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HELMSTETTER, F. J., D. J. CALCAGNETTI, C. P. CRAMER AND M. S. FANSELOW. *Ethylketocyclazocine and bremazocine analgesia in neonatal rats.* PHARMACOL BIOCHEM BEHAV 30(4) 817–821, 1988.—In three experiments we examined the analgesic potency of kappa opioid receptor agonists in 2- and 16-day-old rats. Ethylketocyclazocine (1–50 mg/kg) produced similar dose- and time-dependent increases in the latency to retract a hind paw from a noxious thermal stimulus in rats of both ages. Bremazocine (0.001–10 mg/kg), a kappa agonist with reported antagonist activity at mu receptors, was also effective in producing analgesia in 2-day-old rats. The dose-effect relationship for bremazocine was nonmonotonic. Bremazocine analgesia (0.1 mg/kg) was reversed by both naltrexone and MR2266, a putative kappa opioid antagonist. These results are discussed in terms of the functional integrity of a kappa analgesic system in the developing rat.

Opioids Ontogeny MR2266 Kappa receptor Naltrexone Development Rat Analgesia Hotplate

CONSIDERABLE evidence now exists indicating that opioid receptors are morphologically and functionally subdivided into populations that preferentially bind products of one of three main precursor molecules [1]. Ontogenetic variation has provided further support for heterogeneity in the rat. The density of high-affinity binding to each of the three major opioid receptor subtypes as well as sensitivity to cation and nucleotide regulation display differing patterns of postnatal development [16,22]. After birth, the density of mu sites first decreases between Days 0 and 4 and then rapidly rises between Days 7 and 14, reaching an asymptote twice that of either kappa or delta receptors by Day 28. Delta selective sites are nearly absent at birth and appear slowly during the first two weeks, also reaching their maximum density on or around Day 28. Kappa receptors, on the other hand, are present at birth and their density does not appear to increase significantly over the course of development [16,17].

While the existence of multiple selective binding sites has been demonstrated in neonates, the functional status of these sites may well differ from that seen in the adult. The possibility exists, for example, that while receptors in the neonate are able to bind exogenous ligands, the efficacy of these sites in modulating events at the cellular or behavioral level is a product of some additional maturational process. The existence and distribution of opioid peptides as well as relevant metabolic processes may display ontogenetic profiles independent of receptors [10]. There is some evidence, however, for covariance between the behavioral effects of opiates and the amount of high affinity receptor binding observed in vitro. For example, rats respond to low doses of morphine, a primarily mu agonist, as early as Day 1 postpartum but do not develop morphine tolerance until after about Day 14 [6]. This phenomenon seems to correlate well with the time course of changes in mu receptor binding. While the density of bound kappa receptors in vitro may not change dramatically from birth to adulthood, the ability of compounds acting as these sites to provoke changes in behavior characteristic of the adult may well be determined by other factors. For example, the analgesia produced in infant rats by ketocyclazocine, a primarily kappa agonist, may be dependent on the type of stimulus used to measure pain thresholds and the body region tested, as well as on the age of the animal [7].

The present experiments were designed to characterize the ability of two opioid agonists presumed to act primarily at the kappa receptor, to produce analgesia against a noxious thermal stimulus in infant rats.

EXPERIMENT 1

In the first experiment the increase in thermal pain thresholds produced by four doses of ethylketocyclazocine (EKC), a prototypic kappa agonist, was determined in 2- and 16-day-old rats. While kappa binding should remain relatively stable across this two week period, the in vitro data predict profound differences in both delta and mu binding in rats of these ages.

METHOD

Subjects

Long-Evans hooded rats directly descended from stock obtained from Blue Spruce Farms (Altamont, NY) served as subjects. All animals were bred and maintained in the Dartmouth Psychology vivarium on a 14:10 light:dark cycle with ad lib access to high protein lab chow (Agway) and tap water. Beginning 2 to 9 days prepartum, pregnant females

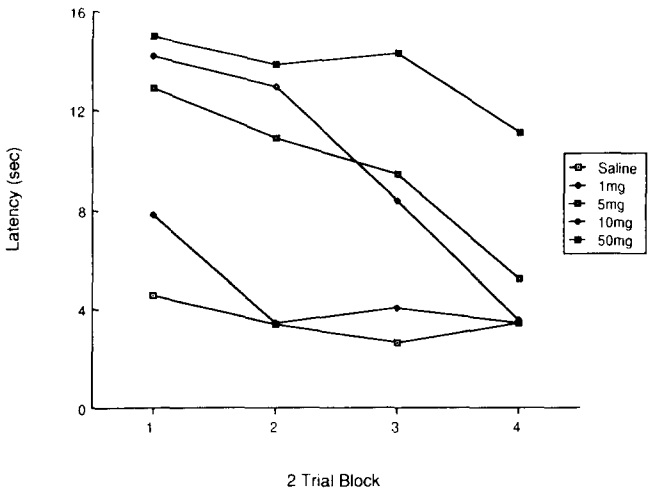

FIG. 1. Dose response and time course of EKC analgesia in 16-day-old rats. Animals were injected IP and the latency to retract a hind paw from a 51°C hotplate was determined over the 160 min period following injection. Each data point represents the mean of two consecutive hotplate tests conducted at 20 min intervals.

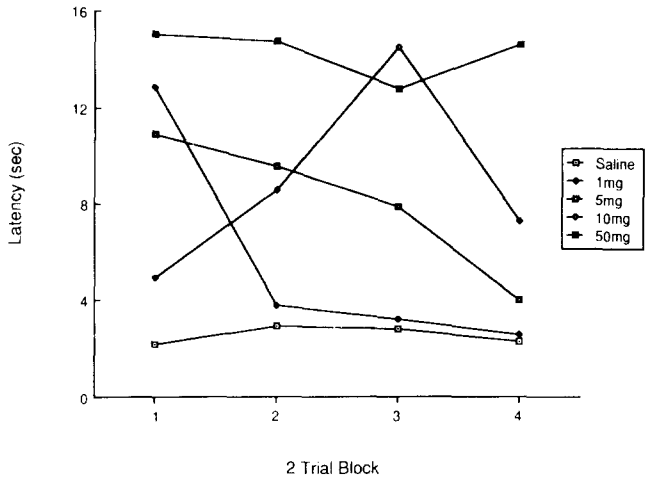

FIG. 2. Dose response and time course of EKC analgesia in 2-day-old rats. Subjects were treated as in Fig. 1. Each data point represents the mean of two consecutive hotplate tests conducted at 20 min intervals.

were individually housed in plastic cages with pine shavings provided for bedding. Cages were checked late each afternoon for births; any pups discovered at that time were considered born that day (Day 0). Litters were culled to 10 pups on the day after birth. A total of 34 rats weighing 30–32 g were tested on Day 16 postpartum. An additional 20 rats weighing 7.5–9.5 g were separated and tested on Day 2 postpartum.

Materials and Procedure

EKC (a gift from Dr. W. Michne, Sterling Winthrop) was dissolved in heated isotonic saline at concentrations of 0.01, 0.05, 1.0 and 5.0 mg/ml. Subjects in each of the four drug groups were given a single IP injection of the appropriate solution in a volume of 10 ml/kg body weight and thus received 1, 5, 10 or 50 mg/kg of EKC. Control animals received a 10 ml/kg injection of isotonic saline.

Animals were separated from their dams, weighed, and marked with a felt tipped pen for identification. Members of each litter were randomly assigned to treatment conditions and retained in groups in small plastic tubs with pine chip bedding. These tubs were maintained at approximately 38°C with a heating pad throughout the experiment. Tests for pain sensitivity began 20 min after drug injection. Analgesia was determined by recording the latency for the animal to retract a paw that was placed in contact with a copper hotplate (Model 35-D IITC, Inc. Landing, NJ) set at 51°C. The experimenter gently held the pup in one hand and slowly lowered it over the hotplate until one hind paw rested firmly on the copper surface. If an animal failed to retract its paw within 15 sec, the trial was terminated. This procedure was repeated for each animal at 20 min intervals, alternating the paw (left or right) being tested each time. Each rat was tested a total of eight times in this manner, thus providing assessment of EKC-induced antinociception from 20 to 160 min after drug injection.

RESULTS

Data for the 16-day-old animals are reported first. Figure 1 depicts the magnitude and time course of EKC analgesia. Each data point in the figure represents the group averaged of a single contiguous pair of left and right paw tests conducted at 20 min intervals from 20 to 160 min after injection. All statistical analyses were performed on individual scores averaged in this manner. As can be seen in the figure, EKC-treated rats evidenced dose-related increases in paw lift latencies relative to controls, with animals in the 10 and 50 mg/kg groups approaching the cutoff of 15 sec early in the test period. This initial elevation in latency decreased in a dose and time dependent manner. By 160 min after injection, response latencies of the 1, 5, and 10 mg/kg group were similar to those of controls. In general, 16-day-old rats given higher doses of EKC exhibited increased latencies on the hotplate for longer periods of time after injection.

These observations were confirmed by the results of a repeated measures analysis of variance (ANOVA) that indicated a reliable Drug × Trial interaction, $F(12,87)=8.98$, $p<0.001$. Planned comparison within the drug factor confirmed the linear dose response relationship, $F(1,29)=351.92$, $p<0.001$. Means within the Drug factor for all but the 1 mg/kg group differed reliably from saline using Scheffe's procedure [8].

As can be seen in Fig. 2, the analgesia produced by EKC in rats tested at two days of age was somewhat similar to that seen in 16-day-olds. Analgesia in the 1 and 5 mg/kg groups tended to decrease as a function of time over the 160 min period from injection to the last test trial. However, analgesia in the 10 mg/kg group did not reach its peak until approximately 2 hr after injection.

A Drug × Trial repeated measures ANOVA indicated a reliable interaction between drug treatment and time of testing, $F(12,45)=7.86$, $p<0.001$. Planned comparisons between group means collapsed over trials indicated a linear dose response relationship, $F(1,15)=176.23$, $p<0.001$. As was the case for 16-day-old rats, when scores for each treatment

group were averaged over the entire testing period, all but the 1 mg/ kg group differed significantly from saline using Scheffe's procedure.

Comparison of Figs. 1 and 2 would indicate that, apart from some differences in the time course of EKC's effects at certain doses, its analgesic potency is quite similar in 2- and 16-day-old rats.

EXPERIMENT 2

While in vitro studies have indicated that EKC preferentially binds to kappa receptors, a number of reports have indicated that the drug may also possess some degree of agonist activity at other opioid binding sites [4,25]. Therefore, the exclusive use of EKC, or similarly ketocyclazocine [2,7], does not rule out the possibility that analgesia after administration of these drugs may be due to activation of nonkappa opioid receptors. Bremazocine is a benzomorphan analogue that binds with reasonable selectively to the kappa receptor and functions as a potent analgesic in adult rats when peripherally or centrally administered [12–14]. It is not cross-tolerant with morphine, produces little respiratory depression, and substitutes for other kappa agonists in operant drug discrimination procedures [12, 20, 24]. In isolated tissue preparations, bemazocine is 20 to 30 times more potent as an agonist than is ketocyclazocine [14]. This compound may be unique among currently available kappa selective agonists in that is has been shown, pharmacologically and behaviorally, to possess antagonist-like properties at the mu receptor [5, 13, 24]. Since this drug binds to both mu and kappa receptors acting as antagonist and agonist, respectively, bremazocine's ability to produce analgesia in the neonate would argue for the functional integrity of a kappa analgesic system and against the possibility that the effects of EKC are due solely to activity at mu receptors. The second experiment examined the effects of bremazocine on 2-day-old rats.

METHOD

Subjects

A total of 78 Long-Evans rats, maintained as described above, served as subjects. Animals weighed 5.5–9.5 g at the time of separation and testing on Day 2 postpartum.

Materials and Procedure

Bremazocine HCl (a gift from Dr. D. Romer, Sandoz) was dissolved at concentrations of 0.0001, 0.001, 0.01, 0.1, 0.25 and 1.0 mg/ml in isotonic saline to which a few drops of 1 N HCl had been added. Solutions were then neutralized with NaOH to a final pH of 6.7. Isotonic saline adjusted to the same pH as the drug solutions served as a vehicle control. Rats were separated into seven groups and injected with 0.001, 0.01, 0.1, 1.0, 2.5, or 10 mg/kg bremazocine (N=10 for each group) or saline (N=18) in a volume of 10 ml/kg as described for Experiment 1. All injections, handling and testing were performed as in the first experiment except that animals were now tested a total of four times at 20 min intervals after injection.

RESULTS

A repeated measures ANOVA performed on latency scores produced a reliable main effect for drug treatment, $F(6,71)=24.15$, $p<0.001$, as well as a reliable trial main effect, $F(3,213)=6.06$, $p<0.01$. Paw lift latency tended to decrease comparably in drug treated and control animals over repeated test trials. Subsequent analyses were conducted on scores averaged over time. Figure 3 depicts the dose-response relationship of these means. As described in the figure, planned comparisons between groups with alpha =0.01 indicated that all but the 0.001 mg/kg group were significantly hypoalgesic with respect to vehicle injected animals. The peak effect for bremazocine was observed at the 0.1 mg/kg dose, with higher concentrations producing less analgesia. Indeed, subsequent comparisons indicated that the decrease observed between the 0.1 and 10 mg/kg groups was statistically reliable ($p<0.01$). There was no linear component to the dose-effect relationship, $F(1,71)<1$, while the quadratic term was reliable, $F(1,71)=127.05$, $p<0.001$. Thus, the bremazocine dose-response relationship in 2-day-old rats is significantly nonmonotonic, with doses higher than 0.1 mg/kg tending to be less effective in producing analgesia in this pain test. In addition, the most effective dose of bremazocine tested (0.1 mg/kg) increased response latency to a mean of 8.55 sec. This maximal effect remains well below the 15 sec cutoff.

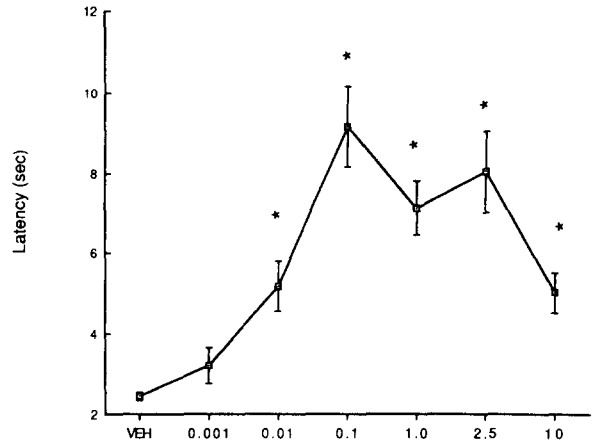

FIG. 3. Dose response for bremazocine (mg/kg, IP) analgesia using 2-day-old rats. Each point on the figure represents the group mean averaged over four consecutive test trials conducted from 20 to 80 min after injection. Asterisks indicate a significant difference from vehicle controls ($p<0.01$). The difference between the 0.1 mg/kg and 10 mg/kg groups is also reliable ($p<0.01$).

EXPERIMENT 3

In the third experiment, the ability of two opioid antagonists to reverse bremazocine induced analgesia in 2-day-old rats was determined. Naltrexone (NTX), like naloxone, displays a much greater preference for mu receptors relative to either delta or kappa receptors [18]. MR2266 ((−) - (1R,5R,9R) - 5,9 - diethyl - 2 - (3 - furylmethyl) - 2′ - hydroxy-6,7-benzomorphan), a putative kappa antagonist, has been demonstrated to be more potent than naloxone in reversing the effects of bremazocine in vitro and in vivo [14]. Three doses of NTX and MR2266 (MR) were administered 15 min after an initial 0.1 mg/kg injection of bremazocine. To the extent that MR is a more effective antagonist at the kappa receptor than is NTX, it should be more effective than NTX in reversing the effects of bremazocine if the analgesia

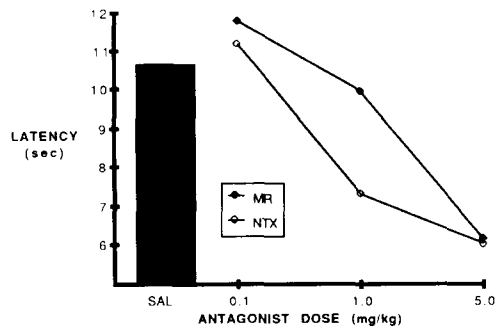

FIG. 4. The ability of MR2266 and naltrexone to reverse the analgesia produced by a 0.1 mg/kg dose of bremazocine in 2-day-old rats. Independent groups of animals were first given bremazocine followed 20 min later by a second injection of an antagonist or saline. Both opioid antagonists reversed the analgesia. Naltrexone was more effective than MR2266 at 1.0 mg/kg ($p<0.05$).

seen in Experiment 2 is produced primarily via the kappa receptor.

METHOD

Subjects

A total of 71 male and female Long-Evans rats maintained as described in the first experiment served as subjects. Animals were separated and tested on Day 2 postpartum.

Materials and Procedure

A single 0.01 mg/ml dose of bremazocine HCl was prepared as described in Experiment 2. Naltrexone HCl (a gift of Dr. J. G. Whitney, DuPont) was dissolved in isotonic saline at concentrations of 0.01, 0.1 and 0.5 mg/ml. MR (a gift of Dr. H. Merz, Boehringer-Ingelheim) was dissolved in saline with the aid of HCl and then neutralized with NaOH to pH 5.6. MR was also prepared in concentrations of 0.01, 0.1 and 0.5 mg/ml. Isotonic saline adjusted to pH 5.6 served as a control.

At the start of the experiment, rats were removed from their dams and weighed as described previously. Each animal first received an IP injection of 0.1 mg/kg bremazocine in a total volume of 10 ml/kg. Fifteen min after this first injection, seven groups of rats received a second injection of either 0.1 (N=9), 1.0 (N=11) or 5.0 (N=10) mg/kg NTX or 0.1 (N=9), 1.0 (N=11) or 5.0 (N=11) mg/kg MR. The last group (N=10) received the vehicle as a second injection. Fifteen min after the second injection testing as described previously began on the hotplate. As in Experiment 2, each animal was tested a total of four times at 20 min intervals, alternating the paw tested each time.

RESULTS

The ability of NTX and MR to reverse the analgesia produced by bremazocine is indicated in Fig. 4. An initial one-way ANOVA performed on latencies averaged over the four test trials indicated significant differences between groups, $F(6,64)=7.32$, $p<0.001$. Subsequent planned comparisons supported a linear dose-reponse relationship for reversal of analgesia by both NTX, $F(1,64)=18.86$, $p<0.001$, and MR, $F(1,64)=14.52$, $p<0.001$. The lowest dose (0.1 mg/kg) of both antagonists failed to reliably influence hotplate latencies. At 5 mg/kg, NTX and MR appeared to be equally effective antagonists (means 6.02 vs. 6.15 sec, respectively). However, at 1.0 mg/kg, NTX was more effective in antagonizing bremazocine's analgesic effects than was MR, $F(1,64)=4.80$, $p<0.04$.

GENERAL DISCUSSION

The present experiments demonstrate that the kappa agonists EKC and bremazocine are able to produce an increase in withdrawal response latency to noxious hindpaw thermal stimulation in rats as early as two days after birth. This increase was dose dependent and, for bremazocine, reversable by the pure opioid antagonists naltrexone and MR2266.

In contrast to the present results, others have reported that analgesia produced by ketocyclazocine does not appear in rats until 10 days after birth when a noxious thermal stimulus is applied to the tail [7]. Furthermore, when the stimulus (water heated to 50°C) was applied to the hindpaws, neither morphine nor ketocyclazocine in doses as high as 10 mg/kg were effective as analgesics in rats ranging in age from 3 to 14 days [7]. These discrepancies are most likely the product of differences in the sensitivity of the test procedure, since morphine in doses as low as 2 mg/kg has been shown to produce robust analgesia in rats as young as one day postpartum using our procedure [3]. Furthermore, morphine's effectiveness in the neonate has been demonstrated regardless of whether the retraction latency of the forepaw, hindpaw, or tail is measured [3].

EKC produced very similar patterns of behavior in both 2- and 16-day-old rats, supporting the idea that its analgesic effects are mediated primarily by the kappa receptor. The density of both mu and delta agonist binding tends to increase during this period, while kappa ligand binding remains relatively stable. If mu or delta receptors were primarily responsible for the analgesia produced by EKC, large quantitative differences between animals at these two points of development might be expected [11]. The results of the first experiment would indicate that, aside from the time to peak effect of one dose (10 mg/kg), 2- and 16-day-old rats respond to the drug comparably (see Figs. 1 and 2). It is possible that the dissimilarities in time course observed between these two groups are due to pharmacokinetic factors, since a number of relevant metabolic processes can only be detected in rats from 3 days after birth [10].

In Experiment 2 we found that bremazocine, which displays both kappa agonist and mu antagonist properties, also produced dose dependent analgesia in 2-day-old rats. Bremazocine's antagonist properties provide additional support for kappa, rather than mu, receptor mediation of benzomorphan analgesia in the neonate. It should be noted that the bremazocine treated group with the longest mean hotplate latency (0.1 mg/kg group at 8.55 sec) was well below the 15 sec cutoff used in this study. Animals of the same age treated with EKC typically exhibited longer latencies and often failed to respond prior to 15 sec (Experiment 1). Both drugs bind not only kappa but also mu and perhaps delta sites. But, while EKC should act as an agonist at each receptor, bremazocine is a mixed agonist/antagonist. Therefore it is possible that EKC has a larger peak effect because of additional mu agonist activity, while bremazocine's effects represent a more purely kappa mediated analgesia. This is consistent with the fact that selective kappa agonists are typically less effective than mu agonists on thermal pain tests such as the one used here [15].

The dose-response curve for bremazocine was reliably

nonmonotonic with doses higher than 0.1 mg/kg tending to be less effective. After informal observation of animals treated with higher doses of the drug we noted that they seemed slightly more active than either low dose animals or controls. It is possible that nonspecific motor activity elicited at higher doses interfered with our ability to measure analgesia in these animals. Interestingly, even pups treated with 50 mg/kg EKC seemed no less, and perhaps slightly more, active than controls—a result very different from the behavioral depression typically seen when rats this age are given similar doses of morphine.

Based on the assumption that MR is a more effective kappa antagonist than is NTX, it was predicted that MR would be more effective in reversing the analgesia produced by bremazocine. However, the two antagonists were almost equipotent in reversing bremazocine's effects. Interestingly, NTX was more effective than MR at the intermediate dose (1 mg/kg). This effect may be due to a difference in general potency rather than selectivity. The majority of evidence for the kappa antagonist activity of MR comes from direct in vitro comparisons with naloxone rather than NTX. While naloxone and NTX are quite similar, both in terms of structure and relative affinity for each receptor subtype [18], NTX is typically more potent in vivo and in vitro [9,23]. While MR is normally more effective than naloxone in antagonizing the effects of kappa agonist in vivo [14,19], at leat one study has reported MR and NTX to be equipotent [21].

In conclusion, we have demonstrated that compounds with kappa agonist properties produce dose- and time-dependent analgesia against a noxious thermal stimulus applied to the hindpaw in rats as young as 2 days of age. The similarity of effects in animals at two different points of development and differing characteristics of a relatively selective and nonselective agonist support the idea that kappa receptors mediate this analgesia.

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