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Placental Opioid-Enhancing Factor (POEF): Generalizability of Effects

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POEF Placenta Amniotic fluid Placentophagia Analgesia Pain Hyperthermia Rat Human Dolphin Parturition Afterbirth Opioids Morphine VSIA

RECENT studies on the nature of Placental Opioid-Enhancing Factor (POEF) have shown that (a) the enhancing effect of ingestion of placenta or amniotic fluid is generalizable to all the opioid-mediated or partly opioid-mediated forms of analgesia tested so far (morphine injection, footshock, mechanical vaginal/cervical stimulation, and that existing during late pregnancy) (12–15); (b) the effect of POEF is not generalizable to nonopioid-mediated analgesia such as that produced by aspirin injection in rats pretreated with the opioid antagonist naltrexone (11); (c) the enhancing effect of ingestion of placenta or amniotic fluid is measurable in a variety of assays (radiant-heat tail-flick latency test, formalin test, hot-water tail-dip test), and is, therefore, not limited to tail mechanisms or thermal pain (11, 13, 14); and (d) amniotic fluid enhances opioid-mediated analgesia whether eaten or delivered directly to the stomach by an orogastric tube, but does not enhance opioid-mediated analgesia when merely seen and smelled but not ingested (12). Therefore, the POEF effect appears to be generalizable across method of opiate- or opioid-mediated analgesia production, type of assay, and method of delivery to the stomach. In addition, the POEF effect appears to be independent of the sensory qualities of placenta and amniotic fluid, and appears to have an effect that is specific to opioid systems.

The work described herein was designed to shed additional light on the generalizability of the POEF effect. Experiment 1 tested for the generalizability of the POEF effect to sex of recipient; Experiment 2 examined whether POEF activity could be detected in afterbirth material of species other than the rat; Experiment 3 tested for the presence of POEF in rat tissue other than placenta; Experiment 4 tested for the effect of POEF when amniotic fluid was injected rather than ingested; and Experiment 5 tested for the effect of POEF on an opioid-mediated process other than analgesia.

EXPERIMENT 1: SEX OF RECIPIENT

To date, our published studies on POEF have been limited to its effect on female rats [e.g., (5, 10–15, 19)]. Although male rats characteristically do not have access to afterbirth substances, we have observed that in the laboratory they ingest placenta if presented with it frequently. We wished to determine whether the physiological system responsible for the POEF effect was present in male rats as well as female rats. Thus Experiment 1 was designed to examine the effect of ingestion of placenta or a control substance (ground beef) on pain threshold in male rats that had received an injection of either morphine sulfate or saline vehicle.

METHOD

Subjects

Eight adult Long-Evans male rats approximately 150 days of age were used. All rats were housed individually in 24.5×

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18 × 18-cm hanging wire-mesh cages in a controlled environment in which the ambient temperature was 22° ± 1°C, the relative humidity was 50%, and a 14 h on/10 h off light cycle (lights on 0500 EST) was maintained. Except where noted, all rats had ad lib access to water and chow (Agway Prolab Rat/Mouse/Hamster Formula 3000).

Apparatus

Pain threshold was assessed by means of a tail-flick latency (TFL) algesiometer similar to that used in other laboratories (4), and described previously (14). Pain thresholds were measured as the number of seconds between the onset of radiant heat and the movement of the tail out of the radiant-heat field. During the TFL tests, rats were restrained in opaque polyvinyl tubes (5 × 21 cm) to which they had previously been exposed. Rats were allowed to habituate to the restrainer for 2 min prior to the first TFL trial. Each TFL score was the mean of the last three of four TFL trials conducted at 30-s intervals.

Prior to the experiment, all rats were habituated to the test cages, the restraint procedure, and the placenta and ground beef substances by repeated exposure (14). Exposure to placenta and ground beef continued until each rat reliably ate 0.5 g in 15 min while housed in the test cages.

Procedure

The experiment was a 2 × 2 × 5 repeated-measures design: Drug [morphine (3 mg/kg, IP); saline (0.9%, 1 ml/kg, IP)] × Enhancer [placenta (2, or approximately 1 g); beef (1 g)] × Time of testing (baseline, 0, 30, 60, and 90 min after ingestion). Each rat was tested in each Drug × Enhancer condition, with one week separating the tests. The order of the four conditions was balanced so that no rat received morphine two weeks in a row. A 3-mg/kg dose of morphine was used because placenta ingestion had been shown to enhance this dose of morphine in prior studies on female rats (10, 11, 13, 14).

Food was removed from the home cage 2 h before testing. At the beginning of the test, baseline TFL was determined and the injection of morphine or saline was administered. The rats were then moved to their test cages. Fifteen minutes later, placenta or beef was proffered in a tip-proof glass dish and rats were allowed a 20-min ingestion period. Postingestion TFLs were determined at 0, 30, 60, and 90 min after the ingestion period.

Rat placenta and beef were obtained, stored, and presented in a manner identical to that described previously (14).

Experimenters determining TFL, in this and all subsequent experiments, were blind to the treatments administered to the rats.

RESULTS AND DISCUSSION

The results of Experiment 1 are depicted in Fig. 1.

The baseline TFLs did not differ significantly among the groups. The means ranged from 3.58 ± 0.07 s in the (Saline + Beef) condition to 3.87 ± 0.09 s in the (Saline + Placenta) condition, $F(3,28) = 2.46$, $p > 0.05$.

A 3-way ANOVA, with a Greenhouse-Geisser correction, revealed a significant Drug × Enhancer × Time interaction, $F(2,16) = 3.83$, $p < 0.05$. Subsequent probes of the 3-way interaction revealed a significant Drug × Enhancer interaction at 0 min after ingestion, $F(1,23) = 9.29$, $p < 0.01$, and a significant Drug × Time interaction in placenta-fed rats, $F(3,31) = 10.77$, $p < 0.01$. Further probes of these interactions were then conducted by pairwise comparisons (with an adjusted MS_{error} term from the overall analysis).

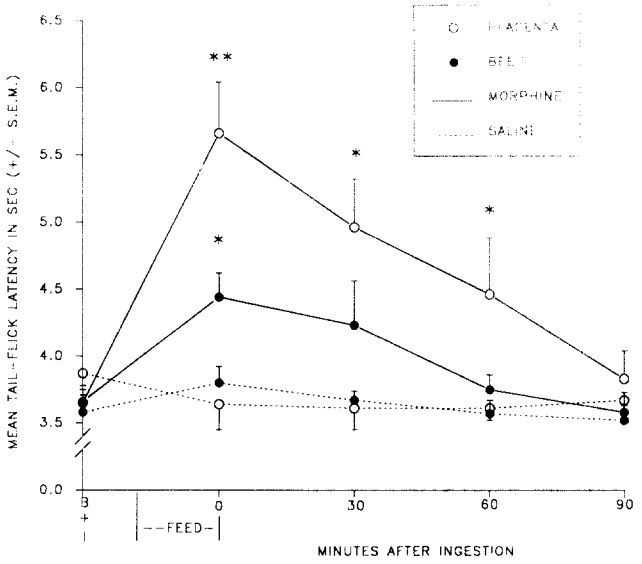

FIG. 1. Mean (± S.E.M.) tail-flick latency (in s) of male rats that ate either placenta or beef after receiving either a morphine (3 mg/kg, IP) or saline (1 ml/kg, IP) injection (n = 8/group). I = injection; * = significantly different from saline groups ($p < 0.01$); ** = significantly different from all other groups ($p < 0.01$).

As expected, morphine injection produced significantly longer TFLs (higher pain thresholds) than did saline injection at 0 min after ingestion: (Morphine + Beef) vs. (Saline + Beef), $F(1,43) = 4.78$, $p < 0.05$; (Morphine + Placenta) vs. (Saline + Placenta), $F(1,43) = 47.88$, $p < 0.0001$.

Among morphine-injected rats, those fed placenta showed significantly longer TFLs (higher pain thresholds) 0 min after ingestion than did those fed beef, $F(1,45) = 13.61$, $p < 0.01$. In saline-treated rats, the effects of ingestion of placenta and beef were the same, $F(1,45) < 1.0$.

Morphine-injected rats fed placenta continued to show significantly longer TFLs (higher pain thresholds) than did saline-injected rats fed placenta at 30 and 60 min after ingestion: (Morphine + Placenta) vs. (Saline + Placenta) at 30 min, $F(1,43) = 21.40$, $p < 0.0001$; (Morphine + Placenta) vs. (Saline + Placenta) at 60 min, $F(1,43) = 8.50$, $p < 0.01$; (Morphine + Placenta) vs. (Saline + Placenta) at 90 min, $F(1,43) < 1.0$.

A Newman-Keuls test indicated that morphine-injected rats fed placenta showed significantly longer TFLs (higher pain thresholds) at 0, 30, and 60 min after ingestion than they did at baseline and 90 min after ingestion. TFLs of morphine-injected rats fed placenta were significantly longer at 0 min after ingestion than at all other test times.

In summary, the results of Experiment 1 show that (a) rats that received morphine but not placenta showed a slight analgesia that was only distinguishable from saline controls at 0 min after the ingestion period; (b) those that received both morphine and placenta showed a significant elevation and prolongation of analgesia that was distinguishable from saline-injected controls at 0, 30, and 60 min after ingestion and that was distinguishable from morphine-injected, beef-fed controls at 0 min after ingestion; (c) those that received both morphine and placenta showed significantly more analgesia at 0, 30, and 60 min after ingestion than at baseline and 90 min after ingestion.

It is clear that the analgesia-enhancing effect of POEF is not limited to female rats. The results of Experiment 1 show conclusively that ingestion of placenta by male rats enhances and prolongs the analgesia produced by a 3-mg/kg injection of morphine. This suggests that the effect of POEF may be a general pharmacological effect rather than one that is limited to the circumstances surrounding parturition in females.

EXPERIMENT 2: SPECIES OF PLACENTA

Parturitional placentophagia is a behavior that is characteristic of most nonhuman mammals. Up to now, however, we have tested for the presence of POEF activity only in rat placenta and amniotic fluid. In Experiment 2, we sought to determine if POEF activity could be demonstrated in afterbirth substances from mammalian species other than the rat, particularly in species not known to engage routinely in parturitional placentophagia (8). Specifically, we examined the effect of ingestion of a preparation of human placenta (Experiment 2a) or of dolphin placenta (Experiment 2b) on pain threshold in female rats experiencing mild opioid-mediated analgesia. Although the decision to test human and dolphin placenta was determined by the availability of these placentas, the characteristic absence of true parturitional placentophagia in humans and the absence or infrequent occurrence of placentophagia in aquatic mammals led us to reason that if POEF activity could be demonstrated in placentas of these species, it would be fair to hypothesize its presence in placentas of placentophagic mammalian species.

EXPERIMENT 2A: HUMAN PLACENTA

In this experiment, we examined the effect of ingestion of a fraction of human placenta on pain threshold in female rats experiencing partly opioid-mediated analgesia induced by vaginal/cervical stimulation (15). To reduce the volume of placenta needed for the procedure, we used standard procedures to separate the human placenta into several fractions. Although we tested each fraction for POEF activity, we report here the results obtained with the active fraction.

METHOD

Subjects

Twelve adult Long-Evans female rats, approximately 120 days old, were used. All rats showed normal estrous cyclicity, as determined by daily inspection of vaginal smears, and were tested only in diestrus. Rats were housed and maintained as in Experiment 1.

Apparatus

Pain thresholds were assessed using the same radiant-heat tail-flick apparatus that was used in Experiment 1. In this experiment, however, vaginal/cervical stimulation (VS) was applied 30 s after determination of baseline TFL, and three additional TFL measures were then obtained at 30-s intervals.

Vaginal/cervical stimulation was applied by removing the back of the restrainer and probing the vaginal cervix with a glass rod protruding from the barrel of a glass, 1-cc tuberculin syringe. The rod was spring loaded and calibrated to deliver a pressure of 75 g (3,15). Stimulation was maintained throughout the 3 final TFL determinations.

A fresh, healthy, human placenta was supplied by Children's Hospital of Buffalo (NY). The placenta was refrigerated within minutes after delivery and was transported to the laboratory and processed within hours. It was kept at 4°C throughout the separation procedures. The placenta was first minced and mixed with cold sucrose (0.25 mM). The placenta/sucrose mixture was then homogenized and filtered through cheesecloth. This filtrate was centrifuged to remove cellular debris, and the cytosol was then passed through a PM30 filter (Amicon) which generally retains molecules with a molecular weight of over 30,000 Daltons. Some of this filtrate was frozen in 0.5 ml samples for subsequent testing. The sucrose control (0.25 mM) was stored and frozen the same as placenta fractions. All fluids were administered at body temperature.

The experimental and control fluids were delivered through an orogastric tube consisting of an 11.5-cm length of PE-160 tubing, fitted to a blunted 16-ga hypodermic needle fitted to a glass syringe. Each rat had been habituated to the intubation procedure (without infusion of fluid) and the restraint procedure five times before testing.

Since a number of different volumes of various placenta fractions were to be tested, a 2-ml sucrose control, which corresponded to the largest volume of placenta fraction we anticipated testing, was used. Ultimately, only 0.5 ml of the PM30 filtrate of human placenta cytosol was needed; a subsequent group of rats was tested on a 0.5-ml volume of sucrose control to eliminate any confound of the results due to stomach distention (10).

Procedure

The experiment used a single-factor design (human placenta fraction vs. sucrose control), with $n=6$ in each group. Vaginal smears were obtained daily; rats were tested only in diestrus, and only after they had demonstrated normal estrous cyclicity.

Food and water were removed 3 h before testing. Baseline TFL was determined first, followed 30 s later by the application of VS and the determination of the VS-TFL. The rat was then immediately intubated and infused with either 0.5 ml human placenta fraction or 2 ml sucrose (and subsequently, 0.5 ml sucrose). Ten minutes later, the postinfusion baseline TFL was determined; 30 s later, VS was applied and the postinfusion VS-TFL was determined.

For each rat, the percent change from baseline TFL was computed for both preinfusion and postinfusion measures. The difference in the percent change from preinfusion to postinfusion TFL was then computed. The median difference in percent change from the preinfusion level to the postinfusion level was computed for each group.

RESULTS AND DISCUSSION

Median tests and Fisher exact probability tests (because of small group sizes) were performed on the differences in percent change from the preinfusion value to the postinfusion value.

There were no significant differences between rats given 2 ml of sucrose and those given 0.5 ml of sucrose ($p>0.05$). Neither the saline-treated nor cytosol-treated rats showed a significant increase in TFL from preinfusion baseline to postinfusion baseline ($p>0.05$). Rats given 0.5 ml human placenta cytosol showed a significantly greater change in pain threshold from the preinfusion value to the postinfusion VS + TFL value (median $= +32.67\%$) than did rats given 2 ml sucrose control (median $= -6.19\%$) ($p<0.05$). Therefore, (a) ingestion of human placenta produced enhancement of vaginal/cervical stimulation-induced analgesia, and (b) the POEF activity was found in that cytosol fraction containing molecules smaller than approximately 30,000 Daltons.

It should be noted again that the POEF activity of human

placenta was demonstrated in rats. Therefore, human POEF must be very similar to rat POEF in order to be able to activate this analgesia-enhancing system in rats.

EXPERIMENT 2B: DOLPHIN PLACENTA

In this experiment, we examined the effect of ingestion of fractions of dolphin placenta on TFL (pain threshold) in female rats experiencing analgesia induced by vaginal/cervical stimulation.

METHOD

Subjects

Sixteen adult Long-Evans female rats, approximately 90 days of age, and having a mean weight of 297 ± 5 g, were used. All rats showed normal estrous cyclicity, as determined by daily inspection of vaginal smears, and were tested only in diestrus. Rats were housed and maintained the same as the rats in Experiment 1.

Apparatus

Pain thresholds were assessed using the same TFL apparatus and the same procedure described in Experiment 1. After Experiment 2a, however, it was determined that the paradigm for testing vaginal/cervical stimulation-induced analgesia could be simplified to eliminate multiple VS-TFL trials within a test. In addition, it was determined that the entire preingestion TFL test could be eliminated. These changes greatly simplified the testing paradigm without sacrificing accuracy or effectiveness. Therefore, in this experiment, 4 baseline TFL measures were obtained 10 min after ingestion. Thirty seconds later, VS was applied and an additional TFL measure was obtained after 10 s of continuous VS.

The procedures for application of VS were the same as in Experiment 2a. In the present experiment, however, the procedural simplifications described above resulted in the administration of considerably less vaginal/cervical stimulation per test. The amount of analgesia resulting from VS depends on a number of factors including the number of repetitions of the tactile stimulation (3, 5, 15, 19); therefore, 125 g VS pressure was needed in Experiment 2b to provide the amount of stimulation equivalent to repeated 75-g stimulations that rats received in Experiment 2a.

A freshly delivered placenta from an Atlantic bottlenose dolphin (*Tursiops truncatus*) was donated by the Niagara Falls Aquarium (Niagara Falls, NY). The placenta and the sucrose control were prepared, processed, stored, and presented the same as was the human placenta in Experiment 2a, except that the filtrate from the PM30 filter was then passed through a YM5 filter (Amicon). The YM5 filtrate, which was administered to the subjects, nominally contained molecules with molecular weights of less than approximately 5,000 Daltons (although this is not an exact cutoff, and molecules of up to about 8,000 Daltons might actually have been present).

All rats were habituated to the restraint and intubation procedures by repeated exposure as in Experiment 2a.

Procedure

The experiment used a single factor design: 0.5 ml dolphin-placenta derivative vs. 0.5 ml sucrose. The 16 rats were randomly divided equally into experimental and control groups. Vaginal smears were obtained daily; rats were tested only in diestrus.

Food and water were removed 3 h before testing. At the beginning of the test, the rat was intubated and infused with either 0.5 ml dolphin placenta fraction or 0.5 ml sucrose. Ten minutes later, the postinfusion baseline TFL was determined; 30 s later, VS was applied and the postinfusion VS-TFL was determined. For each rat, the percent change from baseline TFL was computed.

RESULTS AND DISCUSSION

The percent change from baseline data was analyzed by means of 1-way ANOVA.

The baseline TFLs did not differ significantly between the groups. Mean baseline TFLs were 3.88 ± 0.07 s in the experimental group (dolphin placenta) and 3.90 ± 0.09 s in the control group (sucrose), $F(1,14)<1$.

Rats receiving the YM5 filtrate of dolphin placenta showed a greater increase in pain threshold during VS (57.72 ± 14.84%) than did rats receiving sucrose control (16.24 ± 7.24%), $F(1,14) = 6.31$, $p<0.05$. Therefore, (a) ingestion of dolphin placenta produced enhancement of vaginal/cervical stimulation-induced analgesia, and (b) the POEF activity was found in the cytosol fraction containing molecules smaller than about 8,000 Daltons.

It should be noted that the POEF activity of dolphin placenta was demonstrated in rats. Therefore, dolphin POEF (as well as human POEF) must be very similar to rat POEF in order to be able to activate this analgesia-enhancing system in rats.

EXPERIMENT 3: TYPE OF TISSUE

Presumably, parturient female mammals benefit from ingesting birth materials before and during delivery by experiencing an enhancement of endogenous opioid-mediated (pregnancy-induced) analgesia (13). Therefore, POEF may have a specific role in the parturition of mammals. An alternative, however, is that opioid-enhancing factors may be present in tissues other than afterbirth materials, and may function to enhance analgesia in a number of behavioral circumstances. For example, if opioid-enhancing factors were associated with any of the internal organs, a predator could benefit from ingestion of its prey by experiencing enhancement of opioid-mediated processes related to hunting, predation, or feeding. In Experiment 3, we examined the effect of ingestion of one other type of tissue, liver from pregnant rat donors (the same donors used for placenta and amniotic fluid), on pain threshold in rats experiencing a low level of morphine-induced analgesia.

METHOD

Subjects

Eight adult Long-Evans female rats, approximately 120 days of age, and having a mean weight of 280 ± 5 g, were used. All rats showed normal estrous cyclicity, as determined by daily inspection of vaginal smears, and were tested only in diestrus. Rats were housed and maintained the same as the rats in Experiment 1.

Apparatus

Pain thresholds were assessed using the same TFL apparatus described in Experiment 1. All rats were habituated to the re-

straint procedure and the pregnant-rat liver by repeated exposure as in Experiment 1.

Procedure

The experiment used a single-factor repeated-measures design: 0.5 g pregnant rat liver administered to rats treated with 3 mg/kg morphine sulfate vs. 0.5 g pregnant-rat liver administered to rats treated with 1 ml/kg saline (0.9%). Each rat was tested in both conditions, with one week separating the tests. The order of the conditions was balanced.

Food was removed 2 h before testing. At the beginning of the test, baseline TFL was determined and an injection of morphine or saline was administered. Fifteen minutes later, 0.5 g pregnant-rat liver [an amount equal to the optimum amount of placenta for enhancement of this dose of morphine (9)] was proffered in a tip-proof glass dish. After 30 min, the postfeeding TFL was determined.

Liver from pregnant rats was harvested during the collection of placenta and amniotic fluid from time-bred donors, and was stored, prepared and presented just as the placenta was. The placentas that were collected from the same donors were used in an experiment underway at that time, and were found to contain normal amounts of POEF activity (producing 50%–75% increase from baseline TFL, using analgesia induced by 3 mg/kg morphine).

A 1-way ANOVA was performed on these data. In addition, the data collected from rats receiving morphine and ingesting 0.5 g pregnant-rat liver were compared statistically to data collected previously from identically treated rats receiving 3 mg/kg morphine and ingesting 0.5 g ground beef, and from identically treated rats receiving 3 mg/kg morphine but ingesting no meat.

RESULTS AND DISCUSSION

A 1-way ANOVA revealed that, as expected, morphine injection elevated pain threshold, whereas saline injection did not, $F(1,7)=8.84$, $p<0.05$. However, there were no significant differences found (mean % change from baseline TFL) among the groups [(morphine + pregnant-rat liver) = +16.88% ± 5.34%; (morphine + ground beef) = +12.34% ± 3.97%; (morphine + no meat) = +20.65% ± 17.55%], $F(2,18)<1.00$.

In summary, the results of Experiment 3 show that ingestion of pregnant-rat liver apparently does not enhance opiate-mediated analgesia, at least not the amount of liver given here. This suggests, but does not prove, that POEF is specific to afterbirth materials.

EXPERIMENT 4: INGESTION VS. INJECTION

To date, studies of the enhancing effect of POEF on existing opioid-mediated analgesia have been limited to amniotic fluid or placenta that were either eaten or delivered directly to the stomach by orogastric infusion. In this experiment, we examined the effect of intraperitoneal and subcutaneous injections of amniotic fluid or saline on pain threshold (TFL) of rats experiencing analgesia from an injection of 3 mg/kg morphine sulfate.

METHOD

Subjects

Twenty-four adult Long-Evans female rats, approximately 180 days of age, and having a mean weight of 309 ± 10 g, were used. All rats had previously received one injection of morphine in a prior experiment. All rats showed normal estrous cyclicity, as determined by daily inspection of vaginal smears, and were tested only in diestrus. Rats were housed and maintained as in Experiment 1.

TABLE 1

DIFFERENCE BETWEEN MEDIAN % CHANGE FROM BASELINE TFL FOR AMNIOTIC FLUID TREATMENT AND MEDIAN % CHANGE FROM BASELINE TFL FOR CONTROL TREATMENT AT 10 AND 30 MIN AFTER TREATMENT IN MORPHINE-INJECTED RATS

Treatment Route	10 min After Treatment (25 min after morphine)	30 min After Treatment (45 min after morphine)
Intraperitoneal injection		
Amniotic fluid vs. saline (n=5) (n=4)	−4.61%	−4.47%
Subcutaneous injection		
Amniotic fluid vs. saline (n=4) (n=3)	−1.01%	−1.93%
Orogastric infusion		
Amniotic fluid vs. beef bouillon (n=4) (n=4)	+30.93%*	+26.07%*

*Significant difference between AF treatment and control, $p<0.025$.

Apparatus

Pain thresholds were assessed using the same TFL apparatus described in Experiment 1. All rats were habituated to the restraint procedure by repeated exposure as in Experiment 2a.

Procedure

The experiment was a 2×2×2 design: Route (IP injection; SC injection) × Enhancer [Amniotic Fluid, AF (1 ml); Saline, Sal (0.9%, 1 ml)] × Time of testing (baseline and 10 and 30 min after administration of Enhancer) in rats treated with an injection of 3 mg/kg morphine sulfate (IP). Rats were randomly assigned to one of the four Route × Enhancer conditions.

Food and water were removed 3 h before testing. At the beginning of the test, baseline TFL was determined and an injection of morphine was administered. Fifteen minutes later, amniotic fluid or saline was delivered either IP or SC. Postenhancer TFLs were determined 10 and 30 min later. For each rat, the percent change from baseline TFL was computed for the 10-min and 30-min postenhancer tests.

Due to the small group sizes, median tests and Fisher exact probability tests were performed on percent change from baseline TFL comparing (a) IP AF (n=5) vs. IP Sal (n=4) at 10 min; (b) IP AF vs. IP Sal at 30 min; (c) SC AF (n=4) vs. SC Sal (n=3) at 10 min; and (d) SC AF vs. SC Sal at 30 min. In addition, comparisons were made with data collected previously from morphine-injected rats receiving amniotic fluid (n=4) or beef bouillon (n=4) directly into the stomach by orogastric infusion in an identical paradigm.

RESULTS AND DISCUSSION

Table 1 presents a comparison of the three routes of administration. The tabulated values are the differences between amniotic fluid-treated rats and control rats in median percent change from baseline TFL. Positive values indicate greater enhancement in analgesia in AF-treated rats than in controls.

The median tests performed on the percent change from baseline TFL in rats receiving either IP or SC injections of either amniotic fluid or saline were not significant at either the 10-min or 30-min postenhancer tests ($p>0.05$). The median tests performed on data collected previously from rats infused orogastrically with amniotic fluid or beef bouillon, however, showed a significant enhancement of analgesia at both the 10-min and 30-min postenhancer tests ($p=0.014$ for both tests). Therefore, 1 ml amniotic fluid, injected IP or SC, does not elevate pain threshold in rats treated with 3 mg/kg morphine beyond the level produced by the morphine, whereas the same amount of amniotic fluid administered orogastrically to rats receiving 3 mg/kg morphine significantly enhances the morphine analgesia.

In summary, it appears that the enhancing effect of POEF requires involvement of the gastrointestinal system. Perhaps it activates gastric receptors or is itself activated or manufactured in the stomach by acid or enzymes [see (9) for discussion].

EXPERIMENT 5: EFFECT OF POEF ON MORPHINE-INDUCED HYPERTHERMIA

Our previous research on POEF has focused on the enhancement of opioid-mediated analgesia. To test the generalizability of the enhancing effect of POEF to other opioid-mediated phenomena, we assessed the effect of POEF (in amniotic fluid) on opiate-induced hyperthermia.

Both hypothermia and hyperthermia can be reliably produced by systemic morphine injection (2, 7, 17). The direction of change in body temperature is dependent on the dose of morphine; high doses (i.e., >10 mg/kg, IP) produce hypothermia and low doses (i.e., <5 mg/kg, IP) produce hyperthermia. Since the effect of POEF on morphine-induced analgesia had always been tested on low doses (around 3 mg/kg, IP) of morphine (10, 12, 14), we assessed only morphine-induced hyperthermia in the present experiment.

Body temperature changes were measured before and after treatment with a systemic (IP) injection of morphine (either 0.0, 2.0, 3.5, or 5.0 mg/kg) in combination with ingestion of amniotic fluid (AF) or a control fluid (BB for beef bouillon). Analgesia was also assessed to compare the effect of POEF on analgesia and hyperthermia in the same rat.

METHOD

Subjects

Thirty-two virgin Long-Evans female rats, 70 to 105 days old and having a mean weight of 228 ± 3 g, were used. All rats showed normal estrous cyclicity prior to surgical procedures, as determined by daily inspection of vaginal smears, and were housed and maintained the same as the rats in Experiment 1. Food and water were available ad lib except on test days when they were removed for 3 h before testing and remained unavailable for the initial 80 min of the test period.

All rats were ovariectomized 2 weeks before testing to eliminate the variation in body temperature that occurs over the estrous cycle in the rat (1). Anesthesia during ovariectomy was by injection (0.65 mg/kg, IP) of a 4:1 mixture of ketamine hydrochloride and xylazine hydrochloride (Ketaset, 100 mg/ml, Bristol Laboratories; Rompun, 20 mg/ml, Cutter Laboratories). Ovariectomy was confirmed by the persistence of diestrus-type cells in vaginal smears taken daily during the 2-week recovery period.

Apparatus

Body temperature was measured by rectal insertion (to 6 cm) of a flexible temperature probe (Yellow Springs Instruments, model #402) (16). The probe was connected to a digital thermometer (Keithley Model #866, readable to 0.1°C). The temperature recorded was that at which the thermometer stabilized (10 s without change). The entire procedure lasted approximately 1 min.

AF and BB were collected (prepared), stored, and delivered in the manner described previously (12).

Pain thresholds were assessed using the same tail-flick apparatus described in Experiment 1. All rats were habituated to the restraint and intubation procedure by repeated exposure as in Experiment 2a. All rats were habituated to the procedures used in the measurement of body temperature (insertion of the temperature probe) by repeated exposure before the start of the experiment.

Procedure

The overall design used to assess the effect of AF on morphine-induced hyperthermia was a $2 \times 4 \times 5$ factorial: Drug Dose (0, 2, 3.5, 5 mg/kg MS) × Enhancer (AF, BB) × Postinjection Interval (0, 45, 90, 135, 180 min). Each rat was randomly assigned to 1 of 4 drug conditions and then tested twice, once in the AF condition and once in the BB condition (repeated testing on the Enhancer and Postinjection Interval variables). Fluid infusion was balanced and tests were separated by 2 weeks. Body temperature was taken at time 0 just before the drug injection and then 45, 90, 135, and 180 min after the drug injection. Fluid infusion occurred 15 min after the drug injection.

Pain threshold was measured immediately after the body temperature measurement at T_0 and T_{45}.

RESULTS AND DISCUSSION

Body temperature and pain threshold data were analyzed separately. All comparisons were made by ANOVA with repeated measures and, when indicated, followed by simple-effect probes and pairwise comparisons.

Body Temperature

The results of the analysis of the body temperature data are depicted in Fig. 2.

A 3-way ANOVA (Drug Dose × Enhancer × Postinjection Interval) revealed only a significant Drug Dose × Postinjection Interval interaction, $F(9,82)=4.04$, $p<0.001$ (with a Greenhouse-Geisser correction). No effect of Enhancer was found in any other comparison: Drug Dose × Enhancer × Postinjection Interval, $F(12,112)<1.00$; Enhancer × Postinjection Interval, $F(4,12)=2.12$, $p>0.05$; Drug Dose × Enhancer, $F(3,28)<1.00$; or a main effect of Enhancer, $F(1,28)<1.00$. Therefore, AF ingestion did not affect morphine-induced hyperthermia. In addition, AF ingestion did not affect baseline body temperature or changes in body temperature over time in rats not treated with morphine.

The probe of the significant Drug Dose × Postinjection Interval interaction confirmed that morphine injection produced hyperthermia. The probes, which compared the four drug doses at each postinjection interval, showed that different doses of morphine produced significant differences in body temperature at all postinjection intervals [T_{45}: $F(3,71)=3.91$, $p<0.01$; T_{90}: $F(3,71)=7.77$, $p<0.01$; T_{135}: $F(3,71)=10.65$, $p<0.001$; and T_{180}: $F(3,71)=5.61$, $p<0.01$], but not during the preinjection test [T_0: $F(3,71)<1.00$]. The Newman-Keuls pairwise comparison ($p<0.05$) of the four drug doses at T_{45} showed that rats treated with 5 mg/kg MS and 3.5 mg/kg MS had significantly

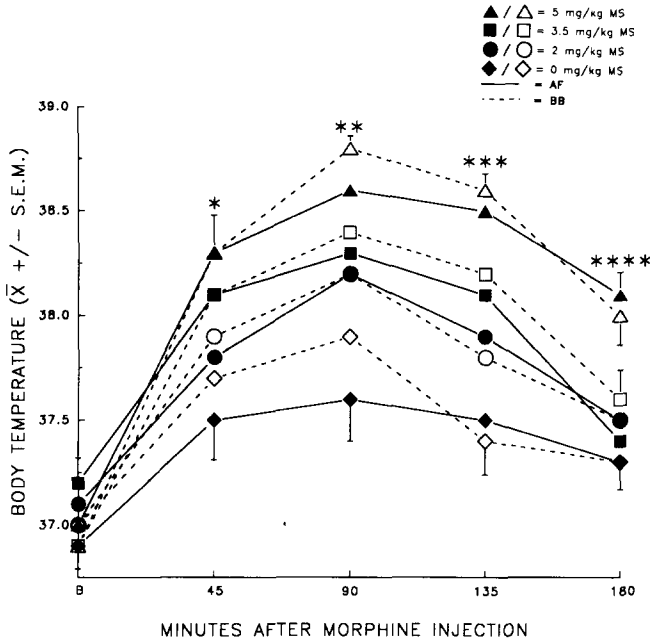

FIG. 2. Mean (±S.E.M.) body temperature (in °C) of virgin female rats before (B) and after injection of morphine (MS) and an orogastric infusion of amniotic fluid (AF) or beef bouillon control (BB). No significant differences between AF and BB infusions were found ($p>0.05$). Differences indicated are for morphine dose only ($p<0.05$). Pain thresholds measured at 45 min were significantly higher in the 2 mg/kg MS + AF group than in the 2 mg/kg MS + BB group ($p<0.05$). *5 mg/kg = 3.5 mg/kg > 2 mg/kg = 0 mg/kg; **5 mg/kg > 3.5 mg/kg = 2 mg/kg > 0 mg/kg; ***5 mg/kg > 3.5 mg/kg > 2 mg/kg > 0 mg/kg; ****5 mg/kg > 3.5 mg/kg = 2 mg/kg = 0 mg/kg.

higher body temperatures than did rats treated with 2 mg/kg MS or 0 mg/kg MS. At T_{90}, rats treated with 5 mg/kg MS had significantly higher body temperatures than did rats treated with any other dose of morphine; at T_{90}, rats treated with 3.5 mg/kg MS had significantly higher body temperatures than rats treated with 0 mg/kg MS. At T_{135}, all drug doses differed significantly from each other in a dose-dependent fashion; the highest body temperatures were found in rats treated with 5 mg/kg MS and the lowest were found in rats treated with 0 mg/kg MS. At T_{180}, only rats treated with 5 mg/kg MS continued to show statistically higher temperatures than did those rats treated with any other dose of morphine.

Small increases in body temperature due to experimental treatment(s) (drug injection, fluid intubation, and/or body temperature and pain threshold measurements) were evident; however, the MS-induced hyperthermia produced by either 2 mg/kg, 3.5 mg/kg, or 5 mg/kg MS was significantly greater, as identified in the comparison of drug doses (see above).

Pain Threshold

The effect of AF ingestion on pain threshold was assessed by separate 2-way ANOVAs performed on each drug dose. A significant effect of Enhancer on pain threshold was found only in the analysis of the 2 mg/kg MS drug dose [Enhancer × Postinjection Interval at 2 mg/kg MS: $F(1,7)=5.67$, $p<0.05$; 0 mg/kg MS: $F(1,7)<1.00$; 3.5 mg/kg MS: $F(1,7)<1.00$; 5 mg/kg MS: $F(1,7)<1.00$]. The probe of the significant interaction found that pain thresholds of rats in the 2 mg/kg MS + AF group increased significantly after drug and fluid treatment [T_0 vs. T_{45} in AF-treated rats: $F(1,16)=10.00$, $p<0.01$], whereas pain threshold of rats in the 2 mg/kg MS + BB group did not increase significantly after the drug and fluid treatment [T_0 vs. T_{45} in BB-treated rats: $F(1,16)<1.00$]. So, whereas 2 mg/kg MS alone did not produce a detectable change in pain threshold itself at T_{45} (no analgesia in BB control rats), a statistically significant increase in pain threshold was obtained when 2 mg/kg MS was combined with AF ingestion. Therefore, AF ingestion enhanced the effect of 2 mg/kg MS on analgesia.

In rats treated with either 3.5 mg/kg MS or 5 mg/kg MS, no effect of Enhancer on pain threshold was found [main effect of Enhancer at 3.5 mg/kg MS: $F(1,7)<1.00$; 5 mg/kg MS: $F(1,7)<1.00$], although both doses produced analgesia [main effect of drug at 3.5 mg/kg: $F(1,7)=9.46$, $p<0.02$; 5 mg/kg MS: $F(1,7)=11.89$, $p<0.001$]. This is consistent with our previous finding that the effect of AF ingestion on analgesia is not linear (10, 13, 19).

AF ingestion enhanced morphine-induced analgesia, but did not modify morphine-induced hyperthermia. These results show that the effect of POEF on opioid-mediated analgesia is not generalizable to all other opioid-mediated phenomena and suggest that POEF action does not extend to the neuroanatomical and/or neurochemical mechanisms involved in opiate-mediated hyperthermia.

The neuroanatomical substrate mediating morphine-induced hyperthermia has not been dissociated completely from that substrate mediating morphine-induced analgesia (20,23), and direct injection of morphine into one site, the periaqueductal gray, leads to both opioid-induced hyperthermia and analgesia (21). Since unique neuroanatomical sites at which opioids act to induce hyperthermia and analgesia have not been isolated, a differential effect of POEF on specific areas of the brain does not seem probable. The absence of different neuroanatomical sites of action for opioid-induced hyperthermia and analgesia does not rule out the possibility that POEF acts to enhance a specific neural pathway, since opioid-induced hyperthermia and analgesia could be mediated by different neural pathways traveling within the same area of the brain.

The neurochemical substrates mediating opiate-induced hyperthermia and opiate-induced analgesia have been characterized by how well receptor-specific opioid agonists mediate the behavioral response. Using this technique, opioids were found to induce hyperthermia only through the μ opioid receptor (18). This type of selectivity was not found in similar analyses of opioid-induced analgesia (6,22). Since amniotic fluid ingestion did not affect morphine-induced hyperthermia, we might speculate at this point that POEF enhancement of opioid activity may be specific to an opioid receptor other than the μ receptor.

GENERAL DISCUSSION

The results of these and past studies provide compelling evidence that the ability of POEF to enhance existing opioid-mediated analgesia is generalizable across type of analgesia, type of assay, gender, and at least three species of donor. Our previous research has shown that ingestion of afterbirth substances clearly offers a specific benefit to the parturient rat. In fact, the finding that production of the substance in question (POEF) may be limited to tissues associated with pregnancy suggests that, in nature, the POEF phenomenon is specifically relevant to parturition. However, the ability to respond to this substance may be widespread among mammals, and therefore may have pharmacological significance and importance well beyond the context of parturitional analgesia enhancement in rats [see (9)].

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