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ENHANCEMENT OF ETORPHINE BRAIN CONCENTRATIONS AND CHANGES IN ETORPHINE–NALOXONE pA_2 VALUES IN MORPHINE-PRETREATED MICE*

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Abstract—We had shown earlier that pretreatment of mice with a single dose of morphine sulfate enhanced the concentration of naloxone in the brain, and, therefore, the effect of this pretreatment on brain disposition of the narcotic agonist etorphine was examined for similar effects. Three hours after pretreatment with morphine, [^{3}H]etorphine was administered subcutaneously, and its brain concentrations were determined as a function of time. Etorphine brain concentrations were higher in morphine- than in saline-pretreated animals at 5, 15, 20 and 30 min. This enhancement of brain concentrations was not associated with a change in the analgesic ED_{50} for etorphine. Morphine pretreatment in mice has been reported by others to increase the affinity of the antagonist receptor site for naloxone, as demonstrated by an increase in the *in vivo* apparent pA_2 value for a morphine–naloxone interaction. In the present study, the morphine pretreatment decreased the etorphine–naloxone apparent pA_2 value in the direction opposite to that observed for the morphine–naloxone interaction. The results are discussed relative to a morphine-induced change in the disposition of etorphine in the brain, or to a morphine-induced alteration in morphine, etorphine and naloxone interactions at agonist and antagonist binding sites.

In a provocative series of papers, Takemori and coworkers [1–3] have shown that a single-dose morphine pretreatment will sensitize mice to the action of the narcotic antagonist naloxone. The magnitude of sensitization was measured *in vivo* in mice as an alteration in the apparent pA_2 value for naloxone, using a morphine-induced analgesia assay. The pA_2 value represents a dose of an antagonist required to double the effective dose of an agonist and approximates the binding affinity of the receptor for the antagonist. The change based on these pA_2 values ranged from a 2-fold increase in potency for naloxone in single-dose morphine-pretreated animals to an 8-fold increase in morphine-tolerant animals. These results were interpreted to represent an increase in receptor binding affinity for naloxone [1]. Although *in vitro* receptor binding assays have not confirmed this hypothesis [4–6], superfusion techniques applied to mouse and rat corpus striatum slices have demonstrated an increased affinity of receptor sites for naloxone in morphine-pretreated and morphine-tolerant animals [7].

Using a similar single-dose morphine pretreatment procedure in mice, Lange *et al.* [8] described a morphine-induced enhancement of naloxone brain concentrations. In addition, morphine-tolerant mice attained significantly higher naloxone concentrations in the brain than morphine-naive mice. The magnitude of the change in naloxone brain concentrations induced by morphine tolerance, however, did not correlate with the large 8-fold increase in naloxone potency reported by Tulunay and Takemori [3] for morphine-tolerant mice. The results of Tulunay and Takemori [2] indicated that narcotic agonists sensitized mice only to narcotic antagonists. The enhancement of brain concentrations of naloxone by morphine pretreatment, however, suggested a relationship between the morphine pretreatment and the increased antagonist potency that was less specific than an increase in the receptor binding affinity for naloxone. The purpose of the present study was to show, therefore, that morphine pretreatment enhanced the brain concentration of a subsequently administered agonist, etorphine. In addition, the effect of this morphine-induced alteration on etorphine brain disposition was evaluated in a pA_2 assay for changes in naloxone potency. Etorphine was selected as the agonist for this study because its lipid solubility [9] and receptor binding affinity [10] were quantitatively more like those of naloxone than were those of morphine.

MATERIALS AND METHODS

Drugs. Sources for the drugs were as follows: naloxone hydrochloride and etorphine hydrochloride, National Institute on Drug Abuse (Rockville, MD); morphine sulfate, Mallinckrodt Chemical

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Works (St. Louis, MO); and [15,16(n)-^{3}H]etorphine (32 Ci/mmole) and [^{14}C-*N*-methyl]morphine (56.4 mCi/mmole), Amersham Corp., Arlington Heights, IL). All compounds were administered subcutaneously (s.c.) in 0.9% sodium chloride at a volume of 0.01 ml/g body weight.

Animals and drug pretreatment schedule. Male, white Swiss–Cox mice (Laboratory Supply, Indianapolis, IN) were used in all experiments. Animal weights ranged from 20 to 35 g. Generally, mice were pretreated s.c. with either 0.9% saline (control group) or with 10 mg/kg morphine sulfate (experimental group). Other agents were given to these animals so that the variable of interest was measured 200 min after the animals were pretreated with saline or morphine.

Measurement of [^{14}C]morphine and [^{3}H]etorphine in the mouse brain. Since we have found previously that a 10 mg/kg, s.c. dose of morphine sulfate given approximately 3 hr before the administration of naloxone would significantly increase the concentration of naloxone in the mouse brain [8], the same morphine pretreatment schedule was used to see if the concentrations of morphine and etorphine might be similarly affected. Both [^{3}H]etorphine (10 μCi/kg; sp. act. 1 μCi/μg) and [^{14}C]morphine (44 μCi/kg; sp. act. 4.4 μCi/mg) were administered s.c. to the saline- and morphine-pretreated groups. At various times thereafter the mice were decapitated. Their brains were removed, and the superficial areas were washed with saline, blotted dry, and then weighed. Each brain was placed in a 7.5 × 1.6 cm glass, screw-topped vial containing 2.0 ml of NCS tissue solubilizer (Amersham Corp.). The capped vials were incubated at 48° for 24 hr in a Dubnoff shaking hot water bath with occasional manual agitation to dissolve the tissue. A 0.3-ml sample was removed and placed in a 7-ml glass liquid scintillation vial containing 5.0 ml of ACS (Amersham Corp.) scintillation fluid. The samples were left at room temperature overnight to allow a chemiluminescent reaction to extinguish. The samples were then counted for the appropriate radionuclide on a Searle liquid scintillation counter (model 300). Quench corrections were made using the external standards ratio feature. From the calculated disintegrations per minute and the specific activity of the administered solution, the concentration of the administered compound in the brain was expressed as pmoles per gram of wet brain weight. Student's *t*-test [11] was employed to compare mean values between groups; significance was set at $P < 0.05$.

Determination of etorphine metabolite disposition to the brain. At a time equivalent to peak analgesia following s.c. [^{3}H]etorphine administration, animals were decapitated, and their brains were removed, washed with saline, blotted dry, and weighed. The brains were individually homogenized in 2 ml of potassium phosphate buffer (0.05 M, pH 7.4) with a Potter–Elvehjem homogenizer. The pH was then adjusted to 8.5 with 100 mg of $NaHCO_3$ and 1 N NaOH. The aqueous phase was extracted with two, 3-ml aliquots of ethyl acetate. Following 30 sec of agitation on a Vortex mixer, the organic and aqueous phases were separated by centrifugation at 2000 *g* for 10 min. The combined solvent extracts were evaporated to dryness with nitrogen and the residue was resuspended in 0.5 ml of ethyl acetate. This procedure resulted in 92 per cent recovery of [^{3}H]etorphine added to a homogenized brain. To check for metabolite disposition in the brain, 50 μl was spotted on a t.l.c. plate (Silica gel 60, EM Laboratories, Inc., Darmstadt, Germany) and developed in cyclohexane–chloroform–diethylamine (50:40:10). The plates were scraped in 1-cm sections, and the radioactivity was determined by liquid scintillation counting.

Measurement of analgesia and determination of pA_2 values. Analgesia was measured with the radiant heat tail flick technique described by D'Amour and Smith [12], with the method of Dewey *et al.* [13] used for the quantitation of tail flick latencies. In a preliminary experiment, etorphine hydrochloride (10 μg/kg) was given s.c. to control mice, and the time course of the analgesic response was measured. This result, together with the previously determined time courses of action for morphine and naloxone [8], was used to fix the parameters of subsequent *in vivo* pA_2 experiments. Naloxone treatment was given 3 hr after the saline or morphine pretreatments, and the measurement of the tail flick latency was performed 20 min later. Treatment with various doses of etorphine (5 or 15 min) or morphine (20 min) was interposed before the tail flick latency response was measured. The purpose of interposing the etorphine or morphine treatment was to determine the ED_{50} for these narcotics at a given dose of naloxone. Each ED_{50} determination required 35–100 mice and included at least four doses of the agonist. The ED_{50} values were calculated using a computerized modification of the procedure of Litchfield and Wilcoxon [14]. The ED_{50} value at a given dose of naloxone was used to calculate a dose ratio, which was defined as the ED_{50} in the presence of the antagonist (naloxone) divided by the ED_{50} without the antagonist. The pA_2 curves were then constructed in accordance with the procedure of Arunlakshana and Schild [15] by plotting log (dose ratio − 1) against -log (dose of naloxone in moles/kg). A line was fitted to the points by the least squares method [11].

RESULTS

In Fig. 1, the concentrations of [^{3}H]etorphine in whole mouse brain are shown for saline- and morphine sulfate-pretreated mice as a function of time following s.c. etorphine administration. The content of radioactivity was significantly higher in the brains of the morphine-pretreated animals at 5, 15, 20 and 30 min. In general, the increase in radioactivity produced by morphine pretreatment represented less than a 20 per cent increase over control values. This increase following morphine pretreatment was similar to that observed for [^{3}H]naloxone brain concentrations [8].

In Table 1, a similar experiment is shown where the concentrations of [^{14}C]morphine in whole mouse brain were measured in saline- and morphine sulfate-pretreated mice. In this case only the brain contents at the time of peak analgesic effect were determined. It is apparent that the morphine sulfate pretreatment did not produce any significant change in the

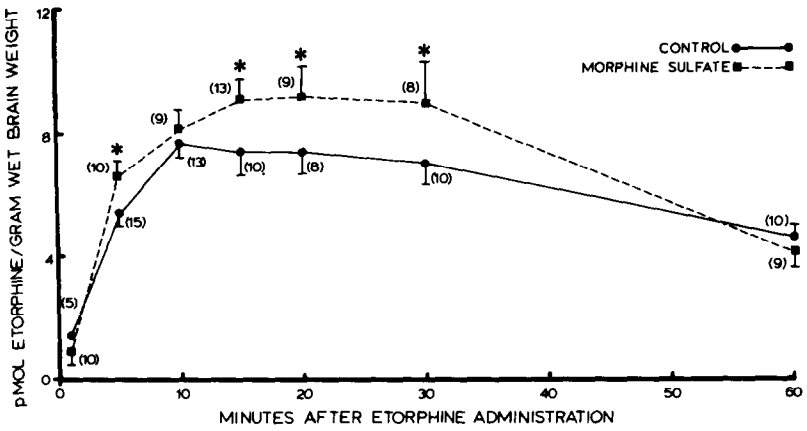

Fig. 1. Enhancement of etorphine brain concentrations by a single morphine sulfate pretreatment (10 mg/kg, s.c.) given 3 hr before [^{3}H]etorphine (10 μg/kg, 10 μCi/kg, s.c.) administration. Controls received 0.9% saline 3 hr before [^{3}H]etorphine administration. Data are means ± S.E.M.; the asterisks indicate a significant difference as determined by Student's t-test ($P < 0.05$). The numbers of animals are enclosed in parenthesis.

disposition of subsequently administered [^{14}C]morphine.

Since it was possible that the change in [^{3}H]etorphine brain concentration following morphine pretreatment was a result of a change in metabolism and disposition of etorphine, we looked for the presence of ^{3}H-compounds other than [^{3}H]etorphine in the brains of morphine-pretreated animals. Using the t.l.c. technique described in Materials and Methods, we were unable to detect any differences between the metabolism of [^{3}H]etorphine in the brains of saline- and morphine sulfate-pretreated animals. With both groups of animals, 91–92% of the total brain radioactivity appeared at the point on the t.l.c. plate associated with the free base of [^{3}H]etorphine standard (R_f = 0.30). Approximately 4 per cent of the total radioactivity remained at the origin, as was observed when [^{3}H]etorphine was added directly to the NCS brain homogenate. No other regions of significant radioactivity were observed.

Since the morphine sulfate pretreatment altered the CNS disposition of etorphine, the analgesic potencies of etorphine in saline- and morphine-pretreated animals were compared. The results in Table 2 indicate that the single-dose morphine pretreatment had no effect on the subsequently determined ED_{50} values of morphine and etorphine. The data for morphine confirm the observation of Takemori *et al.* [1] that there is no residual effect of morphine pretreatment on the subsequent ED_{50} for morphine. Similarly, there was no residual analgesic effect of the morphine pretreatment on the ED_{50} for etorphine. Thus, in spite of the enhanced brain concentrations of etorphine produced by the morphine pretreatment (Fig. 1), no significant alteration in the ED_{50} for etorphine was observed.

Next, experiments were performed to assess the possible consequence of the morphine-induced enhancement of etorphine brain concentrations, by evaluating the naloxone pA_2 values against etorphine analgesia (hereafter called etorphine–naloxone pA_2) in mice. To conduct *in vivo* apparent pA_2 assays, the time to maximal effect for both the agonist and antagonists should be known. This is particularly true for the narcotic antagonist, since the apparent pA_2 value reflects the concentration of the antagonist at the receptor site [16]. In our previous study [8], we found naloxone to have its maximum antagonist activity against morphine analgesia 20 min after s.c. administration, using the acetic acid stretch test in mice. A similar time course of naloxone activity against morphine was obtained using the tail flick analgesia assay in mice. These observations confirm

Table 1. Brain concentration of [^{14}C]morphine after a morphine sulfate pretreatment

Pretreatment*	N	Morphine† (pmoles/g wet brain weight)
Saline	10	290 ± 11
Morphine sulfate (10 mg/kg, s.c.)	10	300 ± 21

* Pretreatment was administered 3 hr before injection of the test dose of [^{14}C]morphine.

† Brain levels of [^{14}C]-radioactivity 20 min after s.c. administration of [^{14}C-*N*-methyl]morphine sulfate (10 mg/kg, 44.0 μCi/kg). Results are expressed as morphine free base (means ± S.E.M.).

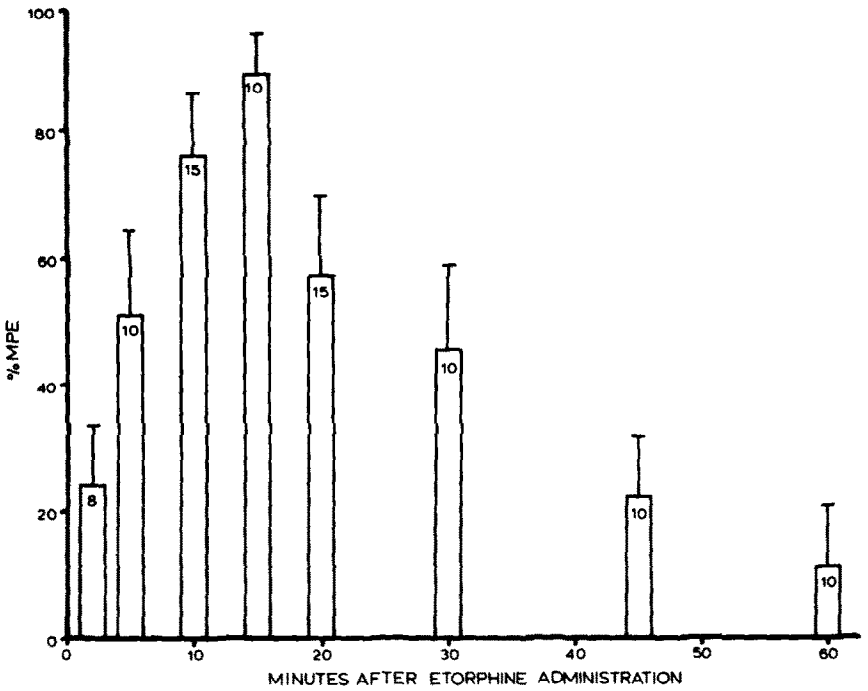

Fig. 2. Time course of antinociceptive activity, expressed as per cent maximum possible effect (MPE) in the tail flick assay (mean ± S.E.M.), following a 10 μg/kg, s.c. dose of etorphine HCl. The number of animals at each dose are contained within the bars.

data previously reported by others [1, 17–19]. In Fig. 2, the time to maximum analgesia for s.c. administered etorphine was found to be 15 min. As a result, the time of drug administration prior to the measurement of analgesia was as follows: 15 min for etorphine, 20 min for morphine [8], and 20 min for naloxone.

Table 3 illustrates the effect of a 3-hr pretreatment with morphine on the pA_2 of naloxone measured against morphine analgesia (morphine–naloxone pA_2). In this set of experiments, the pA_2 line was shifted to the right, and the extrapolated pA_2 value changed from 7.88 in controls to 8.12 in the morphine-pretreated mice. This increase of 0.24 log units in the pA_2 value of naloxone was equivalent to an 80 per cent increase in naloxone potency. These data confirm the earlier observations of Takemori *et al.* [1], who observed a shift of the pA_2 value of naloxone from 6.96 in control animals to 7.30 in morphine-pretreated animals. Both Takemori's data and our observations suggest that approximately a 2-fold increase in naloxone potency occurred as a result of the morphine sulfate pretreatment. Our animals, however, appeared to be more sensitive to the activity of naloxone (higher control pA_2 value) than those used by Takemori and others [1, 17, 20–22].

A similar measure of the apparent pA_2 value of naloxone was performed using etorphine in place of morphine as the test agonist in the analgesic assay. Table 4 shows the results of this experiment, in which etorphine was administerd 15 min before the measurement of tail flick latency in mice. Note that

Table 2. The ED_{50} values of morphine sulfate and etorphine HCl after a morphine sulfate pretreatment

	ED_{50} (95% confidence interval)		
Pretreatment*	Morphine sulfate† (mg/kg, s.c.)	Etorphine HCl‡ (μg/kg, s.c.)	Etorphine HCl§ (μg/kg, s.c.)
Saline	4.5 (3.6–5.7)	3.2 (2.5–4.1)	1.9 (1.1–2.8)
Morphine sulfate (10 mg/kg, s.c.)	4.5 (3.2–6.4)	2.9 (2.3–3.7)	1.8 (1.0–2.9)

* Pretreatment was performed 200 min prior to the measurement of the tail flick response; the treatments with the agonists for the ED_{50} determinations were interposed as indicated below.

† The ED_{50} was determined 20 min after morphine administration.

‡ The ED_{50} was determined 5 min after etorphine administration.

§ The ED_{50} was determined 15 min after etorphine administration.

Table 3. Apparent pA_2 value for morphine–naloxone interaction after a morphine sulfate pretreatment

Pretreatment*	Dose of naloxone HCl†	ED_{50} of morphine sulfate‡ (95% confidence interval)	Apparent pA_2 value (95% confidence interval)	Slope (mean ± S.E.M.)
Saline	0.025	23.5 (13.7–40.1)	7.88 (7.56–8.18)	−0.87 ± 0.11
	0.100	68.5 (42.1–111.4)		
	0.500	297.8 (197.3–449.5)		
	2.00	835.2 (445.6–1565.5)		
Morphine sulfate (10 mg/kg, s.c.)	0.025	47.0 (27.0–81.8)		
	0.100	114.1 (91.8–141.8)	8.12 (7.97–8.22)	−0.95 ± 0.02
	0.500	495.3 (384.3–638.4)		
	2.00	2,745.7 (1295.5–5819.1)		

* Pretreatment was performed 200 min prior to the measurement of the tail flick response.
† Naloxone HCl (mg/kg) was administered s.c. 20 min before the ED_{50} determination.
‡ Morphine sulfate (mg/kg) was administered s.c. 20 min before the ED_{50} determination.

Table 4. Apparent pA_2 value for etorphine–naloxone interaction after a morphine sulfate pretreatment

Pretreatment*	Dose of naloxone HCl†	ED_{50} of etorphine HCl‡ (95% confidence interval)	Apparent pA_2 value (95% confidence interval)	Slope (mean ± S.E.M.)
Saline	0.05	17.5 (9.6–32.0)		
	0.10	25.5 (19.6–33.0)	7.21 (6.86–7.61)	−0.90 ± 0.11
	0.20	37.2 (29.4–47.1)		
	0.30	55.5 (46.8–65.7)		
	0.50	89.8 (72.5–111.2)		
	1.00	193.0 (151.1–246.5)		
Morphine sulfate (10 mg/kg, s.c.)	0.10	22.9 (17.9–29.3)		
	0.20	26.0 (13.9–48.4)	6.89 (6.65–7.15)	−1.12 ± 0.07
	0.30	33.5 (23.2–48.7)		
	0.50	95.5 (71.3–127.9)		
	1.00	199.8 (164.6–242.6)		

* Pretreatment was performed 200 min prior to the measurement of analgesia by tail flick latency.
† Naloxone HCl (mg/kg) was administered s.c. 20 min before the ED_{50} determination.
‡ Etorphine HCl (μg/kg) was administered s.c. 15 min before the ED_{50} determination.

the morphine pretreatment did not shift the curve to the higher values (to the right), as would be expected from the morphine–naloxone interaction following morphine pretreatment. Rather, the etorphine–naloxone pA_2 value decreased from 7.21 to 6.89 with the pA_2 line shifting to the left.

Because the brain concentration of etorphine was increased by the morphine pretreatment (Fig. 1), we hypothesized that this factor may have contributed to the shift to the left of the etorphine–naloxone pA_2 value. Since the pA_2 assays were conducted at the time of peak analgesia we wondered whether the same shift in the etorphine–naloxone pA_2 value might be obtained at another etorphine treatment time. Thus, the etorphine–naloxone pA_2 assays were conducted using a 5-min rather than a 15-min interval for etorphine analgesia. Note in Fig. 1 that 5 min after etorphine administration a significant increase in [^{3}H]etorphine brain concentration was already apparent in the morphine-pretreated animals. Since the time of the naloxone administration remained the same (20 min) in the analgesia assay, we did not expect the etorphine–naloxone pA_2 value to change in control animals. As expected, the pA_2 value of 7.14 (slope = 1.18) for the control animals in this experiment (Fig. 3) was not different from that in the previous experiment (Table 4, $pA_2 = 7.21$). Also, the pA_2 value of 7.17 (slope = 1.16) for naloxone in the morphine-pretreated group (Fig. 4) was the same as for the control group (Fig. 3) if only the data points at the higher doses of naloxone (> 6.26 moles/kg, 0.20 mg/kg) were considered. For naloxone doses of less than 0.20 mg/kg (6.26 moles/kg), however, a second line appeared that was shifted to the left and gave an extrapolated apparent pA_2 value of 6.71 (slope = 1.09). This shift of 0.32 and 0.43 log units to the left of the control pA_2 values can be equated to a 2- to 2.5-fold decrease in the potency of naloxone compared to the control situation.

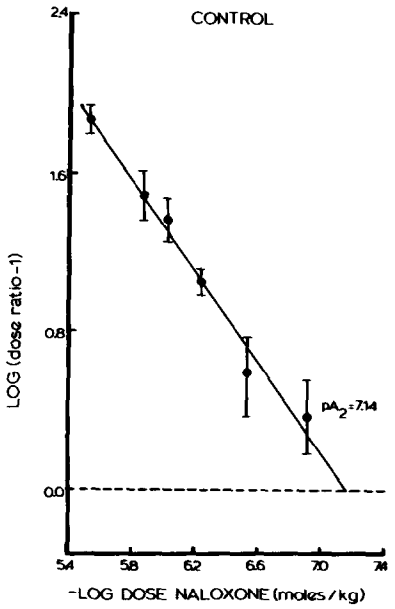

Fig. 3. pA_2 plot for etorphine–naloxone interaction in mice 200 min after saline pretreatment. Etorphine ED_{50} values for analgesia were measured by tail flick assay 20 min after naloxone and 5 min after etorphine administration s.c. The vertical bars are the 95 per cent confidence intervals.

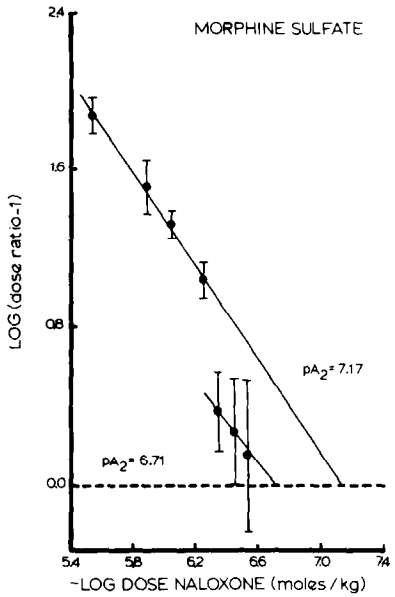

Fig. 4. pA_2 plot for etorphine–naloxone interaction in mice 200 min after morphine sulfate (10 mg/kg, s.c.) pretreatment. Etorphine ED_{50} values for analgesia were measured by tail flick assay 20 min after naloxone and 5 min after etorphine administration s.c. The vertical bars are the 95 per cent confidence intervals.

DISCUSSION

Our data (Table 2) confirm the observations of others [1, 17] that single-dose morphine pretreatment does not affect the ED_{50} of subsequently administered morphine, but that it does increase the antagonist potency of naloxone (Table 3) against morphine-induced analgesia in a pA_2 assay [1, 20–21]. Based on the assumptions inherent in the derivation of the pA_2 relationship, Takemori *et al.* [1] concluded that the change in the apparent pA_2 value of naloxone induced by the morphine pretreatment represented an increase in the receptor binding affinity for naloxone. Furthermore, Takemori [23] postulated that the absence of a change in the ED_{50} for morphine in this situation meant that the antagonist receptor site for naloxone was different from the agonist receptor site for morphine. Thus, it would appear that the morphine pretreatment altered the antagonist receptor site and increased its affinity for naloxone. As proposed, this separation in agonist and antagonist sites would enable changes in antagonist activity without concurrent changes in agonist activity.

Of prime interest in our present study is the observation that a morphine pretreatment decreased the apparent etorphine–naloxone pA_2 value (Table 4, and Figs. 3 and 4). Since it has already been demonstrated in Table 3 that a morphine pretreat-

ment increased the potency of naloxone relative to morphine [1], one might conclude that the use of etorphine as the narcotic agonist reversed this effect of the morphine pretreatment. In quantitative terms, etorphine not only prevented the morphine pretreatment shift of the pA_2 value to the right of the control value by 0.24 log units, but it shifted the pA_2 value an additional 0.43 units to the left of the control value. The total effect of using etorphine as the test analgesic could then be as large as 0.67 log units (0.24 + 0.43), which would represent a 4.7-fold decrease in the relative potency of naloxone. How these quantitative changes in apparent pA_2 values following morphine pretreatment relate to receptor function becomes a difficult question, in the face of the differential binding characteristics of the receptor systems that have been described by others [24–28].

A factor of concern in our data was the discontinuity in the etorphine–naloxone pA_2 curve following a morphine pretreatment. This only occurred when a 5-min interval for etorphine analgesia was used. Clearly, the 5-min interval was too short to allow either a maximal analgesic effect or a maximal brain concentration to develop. From this, one might conclude that the obvious nonequilibrium situation between the receptor and etorphine resulted in the abrupt break in the pA_2 curve. However, a larger issue must be examined before this conclusion can be drawn. The major issue pertains to the variables upon which the pA_2 value is dependent. Furchgott [29] has described one of the basic assumptions underlying the pA_2 derivation as follows: 'When a response to A (agonist) is measured, the concentration of free B (antagonist) in the region of the receptors is in thermodynamic equilibrium with both B combined with receptors and with free B in the bathing or perfusing medium.' Curiously, the reverse assumption is generally not made. In fact, it may not be necessary for the agonist drug added to the system to reach equilibirum with the receptors; it may simply be adequate to control the time or duration of exposure to the agonist in order to conduct the pA_2 assay. This is supported by the mathematical derivation of the pA_2 relationship, in which the agonist units drop out of the derived log (dose ratio -1) expression. Thus, the pA_2 relationship is only dependent upon the assumption that the antagonist is in equilibrium with the receptor sites. This concept is indirectly supported by the work of Tallarida *et al.* [30], who developed a mathematical model to compensate for pA_2 values determined at times other than at peak antagonist concentration.

The lack of a dependent relationship between the time course of the narcotic agonist activity and the resulting apparent pA_2 value for naloxone is supported by our present data. In spite of the considerably lower brain concentrations and analgesic potency for etorphine 5 min after its administration, in comparison to 15 min, there was no difference in the pA_2 values obtained for naloxone in control animals. Unfortunately, we have no explanation for the discontinuity observed in the etorphine–naloxone pA_2 curves, which only occurred after the morphine sulfate pretreatment.

It is important to note that the slopes of the lines representing the data used to calculate the apparent pA_2 values for naloxone were not significantly different from 1. However, since none of the slopes was actually equal to 1, it is possible that a small change in the slope of the pA_2 line was responsible for the alteration in pA_2 values following morphine pretreatment. To evaluate this possibility, the mathematical technique described by Takemori *et al.* [18] was used to correct our pA_2 values for a deviation from a slope of 1. Application of this technique to our data produced little change in our quantitative results and did not alter our interpretations.

Regarding the etorphine–naloxone interactions, Takemori *et al.* [18] reported an etorphine–naloxone pA_2 value of 6.85, which differed significantly from his morphine–naloxone pA_2 value of 7.07. Our results show approximately the same quantitative difference between morphine–naloxone and etorphine–naloxone apparent pA_2 values. In control animals, the etorphine–naloxone apparent pA_2 values of 7.21 (Table 4) and 7.14 (Fig. 3) were lower than the morphine–naloxone pA_2 value of 7.88 (Table 3). Both sets of data demonstrate that the etorphine–naloxone apparent pA_2 value was approximately 0.6 to 0.7 log units lower than the morphine–naloxone apparent pA_2 value. This difference in pA_2 values is interesting, because according to the pA_2 concept, if morphine and etorphine interact with naloxone in the same qualitative fashion at the same receptor site, the naloxone pA_2 should be unchanged irrespective of the agonist used to induce analgesia. Thus, these results may indicate that the interaction of etorphine and naloxone at the receptor was different from that which occurred between morphine and naloxone.

This last interpretation agrees with recent evidence by Childers *et al.* [24] which suggested that the narcotic agonists are represented by two different types of compounds—Group A and B. These two agonist groups were differentiated by their change in *in vitro* binding affinities in the presence and absence of sodium. Using these criteria, etorphine belonged to Group B, and morphine to Group A, agonists. Childers *et al.* [24] were unable to determine whether the differences between Groups A and B agonists were due to changes in agonist interaction at a common receptor or to the existence of two different receptor sites. Similarly, Chang *et al.* [25] reported differential binding sites for narcotic agonists on a regional basis in the brain. Their evidence suggested that different receptor populations existed for the agonist, rather than a single receptor population having different attachment points for the various agonists and antagonists.

A similar argument was offered by Squires and Brastrup [26] who characterized two distinct naloxone binding sites (Types 1 and 2) in rat forebrain. Of particular interest was their observation that etorphine could displace naloxone from a Type 1 binding site but not from a Type 2 binding site. Conversely, morphine displaced naloxone from both Type 1 and Type 2 binding sites. Since the Type 2 binding site appeared not to have a physiologic role [26], the spectrum of agonist affinities for the Type 2 sites may, in part, explain the differences observed in the pA_2 values of naloxone for different competitive agonists. Thus, etorphine would compete

with naloxone at only the Type 1 binding site, leaving all of the Type 2 sites available to interact with naloxone. The absence of an agonist occupancy of the Type 2 site may cause a decrease in the effective concentration of naloxone available to compete with etorphine at the Type 1 binding site. Conversely, morphine interacts at both Type 1 and Type 2 binding sites, which might increase the relative concentration of naloxone available to compete at the Type 1 site. Thus, the lack of etorphine competition with naloxone at the Type 2 binding site may be responsible for the difference in the apparent pA_2 values of naloxone when etorphine is used as the competitive agonist.

Unfortunately, the opposing shifts in naloxone's apparent pA_2 values following a morphine pretreatment are not readily explained using either single or multiple receptor mechanisms. An examination of the two mathematical models developed by Van den Brink [31] for completely separate agonist and antagonist receptor sites (metactoid and metaffinoid systems) offers some insight into the difficulties in using receptor mechanisms to account for the bidirectional shifts in the pA_2 values of naloxone. The metaffinoid system assumes that activation of one receptor system produces an alteration in the binding affinity of the opposing receptor system. The metactoid model assumes that the two receptor–effector systems interact at some cellular level other than at the receptor, so that activation of one system does not alter the receptor–drug interactions of the other system. Thus, in a metaffinoid model, morphine pretreatment would change the affinity of the antagonist binding site for naloxone. In the metactoid model, however, morphine pretreatment would not alter the receptor binding affinity of naloxone, but would alter the potency of the antagonist by changing the sensitivity of the receptor system to activation. In neither type of receptor model is an explanation readily found for the bidirectional change in the apparent pA_2 value of naloxone following a morphine pretreatment. Thus, our observed data presently do not conform to either of these proposed receptor mechanisms.

An additional mechanism can be proposed which integrates our *in vivo* pA_2 observations with the concept of differential binding sites without requiring changes in receptor binding affinity. Using nonequilibrium conditions, it is possible to demonstrate an increased competitive occupancy of receptor sites by an agonist in the presence of an antagonist, following a pretreatment with an antagonist (Stephenson and Ginsborg [32] and Ginsborg and Stephenson [33]). Thus, one might propose the converse situation where the pretreatment of an experimental system with an agonist (morphine) may increase the receptor occupancy of the antagonist (naloxone) in the presence of morphine. This could explain the observation of an increase in the potency of naloxone relative to morphine following a morphine pretreatment. Unfortunately, this mechanism would not explain the observed decrease in the potency of naloxone relative to etorphine following a similar morphine pretreatment. The nonequilibrium mechanism, however, may have some role in the production of the discontinuous pA_2 curve found for low naloxone concentrations when a 5-min interval separates etorphine administration and the analgesia measurement.

The present inability of receptor models to account for the bidirectional shifts in the naloxone pA_2 value following morphine pretreatment has lead us to postulate a dispositional mechanism for the morphine-induced alterations in naloxone potency. Such a dispositional mechanism is particularly attractive in light of our earlier study [8], which showed that a single morphine pretreatment increased subsequent naloxone brain concentrations. This increase would have the effect of increasing the morphine–naloxone pA_2 value, since the morphine pretreatment did not significantly affect subsequent morphine brain concentrations (Table 1). The present finding of an increased etorphine brain concentration following a morphine pretreatment may have similar implications regarding changes in the apparent pA_2 value of naloxone. Thus, the increase in etorphine brain concentrations should have the effect of shifting the etorphine–naloxone pA_2 to smaller values. This effect was observed in Table 4 where the etorphine–naloxone pA_2 value was significantly smaller in the morphine-pretreated animals. Since we had specifically selected etorphine for its lipophilic nature and relatively high receptor binding affinity, we feel that a relationship exists between the test agonist drug potency and the direction of the shift in naloxone's apparent pA_2 value induced by the morphine pretreatment. As of now, we have been unable to determine which component of narcotic potency, drug availability or drug receptor affinity, has the greater role in determining the direction of alteration of the pA_2 value of naloxone. We suspect, however, that both may be involved.

Finally, the ability of a morphine pretreatment to alter the naloxone pA_2 value in mice may have a significant predictive value with respect to the development of narcotic tolerance. If the increase in naloxone's pA_2 value following morphine pretreatment were an index of the tolerance developed to morphine, as suggested initially by Takemori [23], the etorphine–naloxone pA_2 results obtained in our study would mean that cross tolerance to etorphine would not be seen in morphine-tolerant mice. Preliminary results in support of this hypothesis have already been reported [34, 35].

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