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EFFECTS OF ACUTE AND CONTINUOUS MORPHINE ADMINISTRATION ON THE AFFINITY OF GLUTAMIC ACID DECARBOXYLASE FOR PYRIDOXAL 5′-PHOSPHATE

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Abstract—The studies showed that neither morphine nor naloxone at concentrations up to 10^{-3} M exhibited any effect on the affinity of glutamic acid decarboxylase (GAD) for its cofactor, pyridoxal 5′-phosphate (P-5′-P) *in vitro*. Acute administration of morphine sulfate with respect to time course and dose–response studies also failed to modify the affinity of GAD for P-5′-P. In contrast, in mice rendered tolerant to morphine by morphine pellet implantation there was a significant attenuation in affinity of GAD for P-5′-P. The sensitivity of this enzyme for P-5′-P during the course of the development of tolerance to morphine was restored partially either when the morphine was abruptly withdrawn by removing the morphine pellet or when withdrawal was precipitated by naloxone. The recovery of the affinity of GAD for its cofactor was shown to be naloxone dose-dependent. These results further substantiate that the γ-aminobutyric acid (GABA) system may be involved in morphine tolerance and the development of dependence.

Ample evidence has been presented which suggests that γ-aminobutyric acid (GABA), an important inhibitory neurotransmitter in the central nervous system (CNS), may be associated with morphine analgesia, tolerance and physical dependence. Lin *et al.* [1] demonstrated that, in rats made tolerant to morphine, there were increased levels of GABA in the subcortical and hypothalamic areas, compared to control rats. Pharmacologic manipulations of the GABA system, such as slowing the destruction of GABA, blocking its receptor sites, or inhibiting its re-uptake processes have also suggested that the GABA system could play an important role in the effects of morphine [2–4]. The most recent biochemical study from our laboratory demonstrated a significantly increased brain GABA content and decreased glutamate levels following acute morphine administration [5]. Brain glutamic acid dehydrogenase (GAD) and GABA-T activity were not affected. Continuous morphine administration, on the other hand, caused no significant changes in brain glutamate levels. However, there were significant decreases in both GABA levels and GAD activities. Also, the rate of brain GABA accumulation, induced by aminooxyacetic acid in tolerant mice, was slower than in non-tolerant animals.

As the rate-limiting step in GABA synthesis [6], is, potentially, an important site of action for drugs GAD which influence GABA levels. GAD activity requires pyridoxal 5′-phosphate (P-5′-P) as a cofactor for maximal production of GABA [7–10]. GAD activity is evidently sensitive to changes in the level of this vitamin B-6 cofactor, since the enzyme is inhibited by carbonyl trapping agents [11]. It is possible that, during normal or abnormal neuronal function, the affinity of GAD for its cofactor will be altered. Therefore, the present study was designed to investigate the effect of both acute and chronic administration of morphine on the affinity of GAD for its cofactor, pyridoxal 5′-phosphate.

MATERIALS AND METHODS

Male ICR mice weighing 25 ± 3 g (Charles River, Wilmington, MA) were used in various experiments. Animals were maintained on standard laboratory chow and tap water and were housed in a room lighted artifically for 12 hr of the day. The chemicals and their suppliers were as follows: P-5′-P from Sigma Chemical Co., St. Louis, MO; aquasol and [U-^{14}C]glutamic acid from New England Nuclear, Boston, MA; and morphine sulfate from Malinkrodt Chemical Works, St. Louis, MO.

Measurement of GAD activity with different concentrations of P-5′-P. The activity of GAD was determined by measuring the $^{14}CO_2$ formation from [U-^{14}C]-L-glutamic acid according to the method described by Roberts and Simonsen [12], with minor modifications. After removal, brains were homogenized immediately (glass homogenizer) at 4° in 5 ml of ice-cold 0.1 N potassium phosphate buffer (pH 6.5) containing 0.03% GSH. Each assay mixture (total volume of 1.0 ml) contained 100 μmoles of potassium phosphate buffer, pH 6.5, 100 μmoles of L-glutamate (0.9 μCi), 1 μmole GSH and different amounts of P-5′-P as indicated. Two-tenths ml of 1 N hyamine hydroxide solution in methanol was placed in a plastic vial and hung on the rubber stopper in the test tube (18 × 105 mm); the tubes were placed in a Dubnoff shaker at 37°. The reaction was started by injecting 2.0 mg of brain homogenate protein into the assay mixture. After 30 min, 0.2 ml of 4 N H_2SO_4 was injected to stop the reaction and release $^{14}CO_2$. After shaking another 90 min, the contents of the

plastic vial were removed and placed in a liquid scintillation counting vial which contained 10 ml aquasol. The enzyme activity, as μmoles glutamate decarboxylated/30 min/100 mg protein of brain homogenate, was calculated from the $^{14}CO_2$ liberated from [U-^{14}C]-L-glutamate. Protein contents of brain homogenates were determined by the method of Lowry *et al.* [13] with bovine serum albumin as a standard.

In vitro *effects of morphine and naloxone on GAD affinity for P-5′-P.* The GAD activity of brain homogenate was assayed in different concentrations of P-5′-P in the presence of morphine sulfate or naloxone hydrochloride at concentrations of 1×10^{-3}, 1×10^{-4} and 1×10^{-6} M. An equal volume of buffer was used as control.

Time course and dose–response of the effect of morphine sulfate on GAD affinity for P-5′-P. In the time course experiments, GAD activity with different concentrations of P-5′-P was measured at 0, 15, 60 and 90 min after the injection of morphine sulfate, 40 mg/kg, s.c. In the dose–response experiments, mice received morphine sulfate at 10, 20 or 40 mg/kg, s.c. The brain GAD activity at different concentrations of P-5′-P was measured 60 min after the administration of morphine sulfate. The control group received saline for the same period of time.

Effect of the development of tolerance to morphine on the affinity of GAD for P-5′-P. Fifteen mice were divided into three groups. Groups 1 and 2 were made tolerant to morphine by implanting a specially formulated pellet containing 75 mg of the morphine free base subcutaneously for 24 and 72 hr [14] respectively. Group 3 received a placebo pellet implanted as a control. The schedules were arranged so that all groups of animals were killed on the same day. GAD activity was determined as mentioned above.

Effect of abrupt withdrawal on GAD affinity for P-5′-P. In the abrupt withdrawal experiment, each mouse from two groups was implanted for 72 hr with a morphine pellet. The control group was implanted with placebo pellets for 72 hr. One group of the morphine-treated mice had the pellets removed for 24 hr before the mice were killed. The experiment was planned in such a way that all the groups were killed and assayed in the same day. In the abrupt withdrawal experiments, the mice were made dependent by morphine pellet implantation in exactly the same manner that they were made tolerant. At the end of 3 days of pellet implantation, the mice received naloxone hydrochloride ranging from 0.05 to 1 mg/kg, s.c. The control group received the vehicle, saline. The GAD activity at different concentrations of P-5′-P was estimated 10 min after the administration of naloxone hydrochloride.

Statistical tests for significance. Differences between the means of various treatments were evaluated by Student's *t* test. The p values are shown in the figures.

RESULTS

Effect of morphine or naloxone on GAD activity for P-5′-P in vitro. Neither morphine nor naloxone showed any effect on the affinity of GAD activity for P-5′-P *in vitro*. As shown in Figs. 1 and 2, the various concentrations tested of these compounds, ranging from 10^{-6} to 10^{-3} M, showed no significant difference on the affinity of GAD for its cofactor.

Time course and dose–response of the effect of

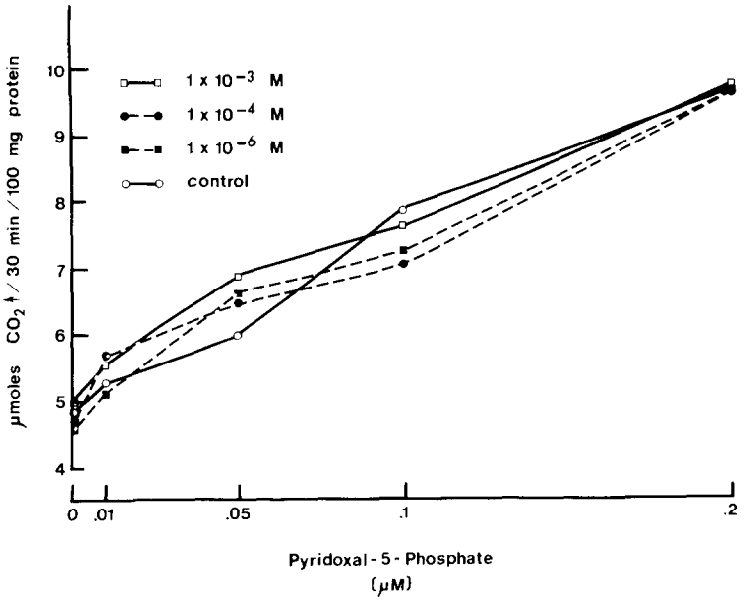

Fig. 1. Effect *in vitro* of morphine sulfate on brain GAD activity. Morphine sulfate was made in such a concentration that only 10 additional μl were injected into the reaction mixture to give the correct final concentration. At least five mouse brains were pooled for each concentration. Each point represents the mean of four assays; S.E.M. for each point is not shown since none of the results shows any significant difference.

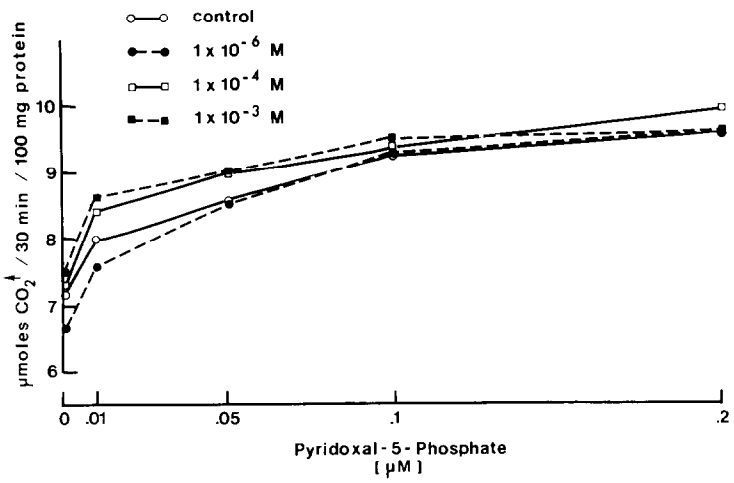

Fig. 2. Effect *in vitro* of naloxone hydrochloride on brain GAD activity. Naloxone hydrochloride was made in such a concentration that only 10 additional µl were injected into the reaction mixture to give the final concentration. Five brains were pooled for each concentration. Each point represents at least four assays.

acute morphine administration on the affinity of GAD for P-5′-P. A time course study (Fig. 3), with the affinity of GAD for P-5′-P being measured at 0, 15, 60 and 90 min after an injection of morphine sulfate, 40 mg/kg, s.c., displayed an apparent stimulation of activity with time, but at only one time point was it significant.

Acute administration of morphine sulfate at three different dosages also was not followed by significant changes in the affinity of GAD for its cofactor. Figure 4 illustrates the effect of an acute injection of morphine sulfate. GAD activity appears to increase with increasing doses of morphine, but not significantly.

Modification of GAD affinity for P-5′-P during the development of tolerance to morphine. In mice implanted with morphine pellets for different periods of time there was a highly significant difference in the response of morphine-treated mice compared to the placebo group, with respect to the effect of varying concentrations of P-5′-P. As shown in Fig. 5, the placebo group had a higher degree of enzyme activity with increasing concentrations of P-5′-P than did the morphine pellet-implanted groups, indicating a greater sensitivity to the cofactor added. Mice implanted with morphine pellets for 1 day exhibited

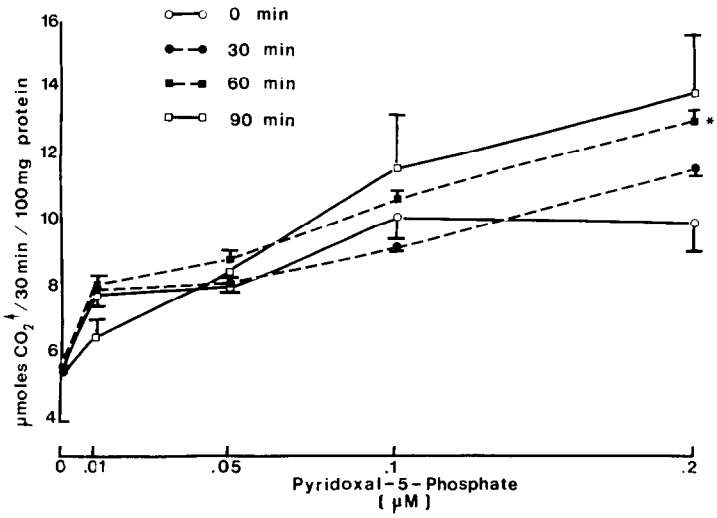

Fig. 3. Effects of acute morphine sulfate administration on brain GAD activity when measured at various times after injection. Mice were injected with 40 mg/kg of morphine sulfate, s.c., and killed at the appropriate time interval. The injections were timed in such a way that all mice were killed and brains removed within 15 min of each other. Five mice were sacrificed for each time interval. Each point represents the mean of four assays. The bracketed vertical lines represent ±S. E. M.

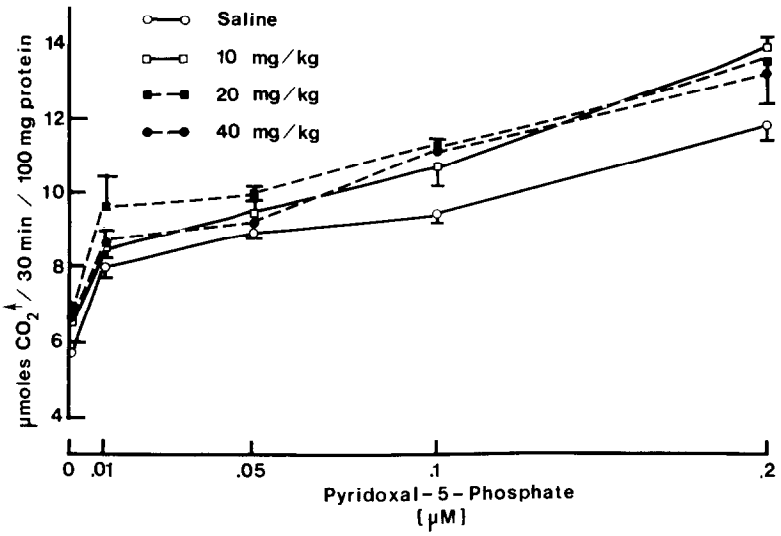

Fig. 4. Effects of acute morphine sulfate administration on brain GAD activity. Mice were killed 60 min after the administration of morphine sulfate, ranging from 10 to 40 mg/kg, s.c. The same volume of saline, the vehicle, was injected s.c. as a control. The bracketed vertical lines represent ± S. E. M.

a significant decrease in GAD affinity for cofactor as compared with control mice implanted with placebo. This alteration of GAD affinity for cofactor was even more evident when results from the 3-day morphine pellet-implanted group were analyzed.

Effect of abrupt withdrawal on GAD affinity for P-5′-P. The affinity of GAD for cofactor was restored toward the normal control after 24 hr of morphine pellet removal. As shown in Fig. 6, the affinity of GAD for its cofactor was largely suppressed after 3 days of morphine pellet implantation. However, in the 3-day morphine-treated group in which the pellet was withdrawn for 24 hr, the GAD activity response was more sensitive to increasing concentrations of cofactor than the 3-day morphine pellet-implanted group. At lower concentrations of P-5′-P the GAD activity in 1-day morphine pellet-withdrawn group was still significantly lower than that of the placebo-implanted group. However, at higher concentrations of P-5′-P, GAD activity approached the level of control mice.

Effect of abrupt withdrawal of morphine by naloxone on GAD affinity for P-5′-P. In mice made dependent on morphine by morphine pellet implantation for 3 days, the abrupt withdrawal of morphine by naloxone tends to restore the affinity of GAD for cofactor. As shown in Fig. 7, administration of naloxone, 0.05 mg/kg, s.c., produced a significant recovery of

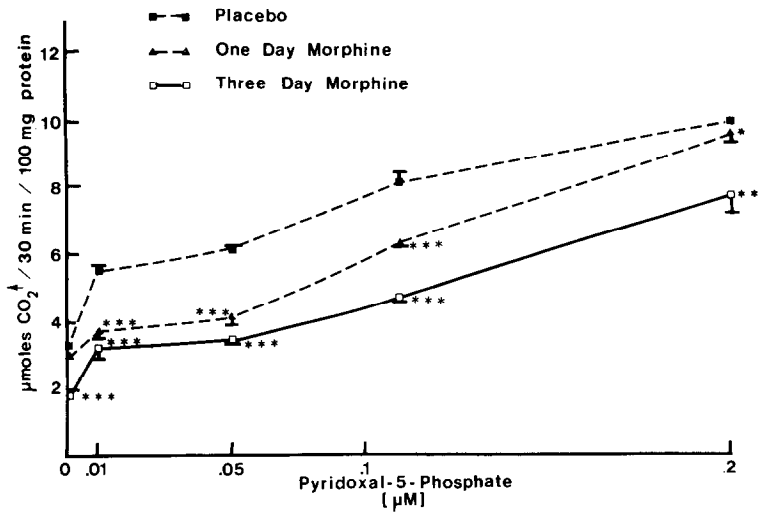

Fig. 5. Effects of chronic morphine administration on brain GAD activity. Each mouse of one group was implanted with a morphine pellet for 24 hr while those of another group were implanted for 72 hr. The control group mice received a placebo pellet for 72 hr. Five mice were used in each group. The vertical lines represent ±S. E. M. Asterisks refer to degree of significance: one asterisk indicates $p < 0.05$, two indicate $p < 0.01$, and three $p < 0.001$.

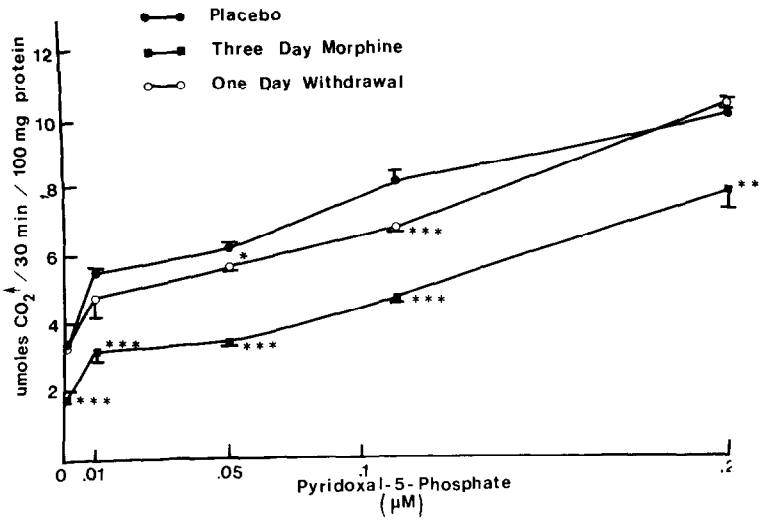

Fig. 6. Effects of abrupt withdrawal of morphine on brain GAD activity. Two groups of mice were implanted with morphine pellets for 72 hr. The control groups received placebo pellets for 72 hr. One of the morphine group had the pellet removed for 24 hr before the enzyme activities were measured. Implantation and withdrawal were scheduled so that all mice were killed on the same day. Five mice were used in each point. The vertical lines represent ±S. E. M. One asterisk indicates $p < 0.05$, two indicate $p < 0.01$, and three, $p < 0.001$.

GAD affinity for its cofactor. The recovery of the affinity of GAD for P-5′-P is dose-dependent. This is evidenced by greater enhancement of GAD activity in mice receiving higher doses of naloxone administration.

DISCUSSION

The present studies demonstrate that neither morphine nor naloxone exhibited any effect on the affinity of GAD activity for its coenzyme, P-5′-P, *in vitro*. Acute administration of morphine sulfate with respect to time course and dose–response studies also failed to modify the affinity of GAD for P-5′-P. In contrast, in mice continuously administered morphine by pellet implantation for different periods of time, there was a significant attenuation in affinity of GAD for P-5′-P. The sensitivity of this enzyme for P-5′-P during the course of the development of tolerance to morphine was restored partially either when the morphine was abruptly withdrawn by removing the morphine pellet or when withdrawal was precipitated by naloxone. The recovery of the affinity of GAD activity for its cofactor was shown to be naloxone dose-dependent.

In our recent studies [5] acute morphine administration resulted in a significantly increased brain GABA content and decreased glutamate levels.

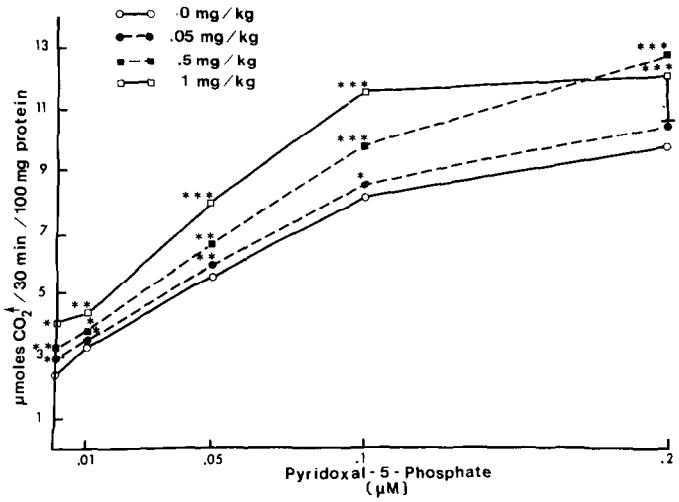

Fig. 7. Effects of various doses of naloxone on dependent mouse brain GAD activity. Mice were made dependent on morphine by implanting pellets for 72 hr, after which each group was injected s.c. with various concentrations of naloxone and killed 10 min later. Five brains were used for each point. The vertical lines indicate ±S. E. M. of four assays. One asterisk indicates $p < 0.05$, two indicate $p < 0.01$, and three, $p < 0.001$.

Neither brain GAD nor GABA-T activity was affected. Continuous morphine administration, on the other hand, caused a significant decrease in both GABA level and GAD activity. Also, the rate of brain GABA accumulation induced by amino-oxyacetic acid in tolerant mice was slower than in non-tolerant animals. The present studies on the effects of acute and continuous administration of morphine substantially support our previous biochemical findings.

Accumulated evidence strongly suggests that the major neuronal system exerting tonic inhibition on pacemaker neurons is the system of inhibitory neurons utilizing GABA as neurotransmitter [6, 15, 16]. GABA neurons are present in the CNS of vertebrate species. The content of GABA in the CNS is much more extensively and relatively more evenly distributed throughout the various brain regions than are the neurotransmitters employed by other neuronal systems e.g. acetylcholine and biogenic amines [15]. Biochemical data also have shown the presence of GAD and GABA in many regions of the vertebrate CNS. As the rate-limiting step in GABA synthesis [6], GAD is a potentially important site of action for drugs which influence GABA levels. It is also possible that during normal or abnormal neuronal functioning, the affinity of GAD for cofactor would be altered. The present studies suggest that the affinity of GAD for P-5′-P was modified during the development of tolerance to and dependence on morphine.

The precise mechanism by which chronic morphine administration altered GAD affinity to cofactor remains to be elucidated. It is possible that chronic morphine administration alters the affinity of GAD for its cofactor, which may inactivate pharmacologically the enzyme which is rate limiting in GABA synthesis. This phenomenon has been reported [17], in that dopamine formation is stimulated by certain conditions and drug treatments which increase the affinity of tyrosine hydroxylase for both its substrate and cofactor. On the other hand, the structure of GAD may be modified during the course of tolerance-dependence development. Consequently, the enzyme activity would be modified. With the newly developed immunocytochemical techniques [18], it is possible to envision effects on molecular levels. Since the isolation and purification of GAD from mouse brain, the antibodies produced in rabbits to GAD obtained from mouse brain were found to cross react with GAD from human brain; microcomplement fixation tests also show the mouse and human enzymes to be quite similar [18]. Work is in progress to carry out the microcomplement fixation tests on GAD obtained from both morphine and control animals. From such tests the chronic effects of morphine on GAD, whether due to a decrease of synthesis or a modification of structure, will be revealed.

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