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DIRECT CORRELATION BETWEEN LEVEL OF MORPHINE AND ITS BIOCHEMICAL EFFECT ON MONOAMINE SYSTEMS IN MOUSE BRAIN

EVIDENCE FOR INVOLVEMENT OF DOPAMINERGIC NEURONS IN THE PHARMACOLOGICAL ACTION OF ACUTE MORPHINE

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Abstract—The pharmacokinetics (uptake and elimination) and pharmacodynamics (biochemical effects on monoamine systems) of morphine in the CNS were investigated concurrently. ICR mice, weighing about 25 g, were injected intravenously with several doses (2.5–80 mg/kg) of morphine. The animals were killed by microwave irradiation (5 kW, 0.6 sec) at 10 and 30 min, and 1, 2, 4, 8 and 24 hr after the injection. The intracerebral levels of morphine and metabolically related substances consisting of monoamines {noradrenaline, dopamine (DA), 5-hydroxytryptamine (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxyphenylacetic acid [homovanillic acid (HVA)], 5-hydroxyindoleacetic acid (5-HIAA), tyrosine and tryptophan} were determined in identical samples by a combination of organic extraction and high-performance liquid chromatography with electrochemical detection. The intracerebral level of morphine was found to depend on the dose injected, and the biological half-life of the drug was estimated to be about 1 hr. The morphine injection (2.5–80 mg/kg) caused significant increases in monoamine metabolites although only slight changes occurred in the concns of parent transmitters. The intracerebral level of morphine was significantly correlated with the ratios DOPAC/DA and HVA/DA ($r = 0.7033$, $P < 0.0001$; and $r = 0.6455$, $P < 0.0001$, respectively). On the other hand, the correlation between the morphine level and 5-HIAA/5-HT was lower than those for DOPAC/DA and HVA/DA. These results suggest that monoamine systems, especially DA, are closely involved in the biochemical effects of morphine. Furthermore, the proposed procedure is demonstrated to be useful as a new approach in biochemical pharmacology, where the direct correlation between the distribution of a drug (pharmacokinetics) and the biochemical effects of the drug (pharmacodynamics) can be measured.

Morphine is an important drug, as an experimental tool, for investigating pain and drug dependence since its pharmacological and toxicological effects include an analgesic action and dependence. It has been shown that morphine affects the biochemical functions of the CNS [1, 2]. Several investigators have recently reported that acute morphine leads to acceleration of the turnover rates of noradrenaline (NA) [3–5], dopamine (DA) [3–7] and 5-hydroxytryptamine (5-HT) [4, 8–12] in the brain. These findings suggest that monoamine systems may be involved in the biochemical and pharmacological effects of the drug. Many studies have demonstrated a direct correlation between the intracerebral concns and behavioral effects of morphine. For example, it has been reported that the analgesic effects of the drug are proportional to the concns in the brain [13, 14]. However, no evidence has yet been reported for a direct correlation between morphine levels and biochemical effects *in vivo*.

The biochemical interest in morphine has led to the development of assay techniques for the drug by various approaches [14–16]. High-performance liquid chromatography with electrochemical detection (HPLC-ECD) has been widely applied for the determination of biogenic amines and their related substances [17, 18]. The detector is sensitive for compounds having a phenolic hydroxy group in their chemical structure. Morphine is such a drug and is reported to be detected by HPLC-ECD [19]. One study [20] has more recently introduced a sensitive and simple procedure for the simultaneous determination of morphine and monoamine-related substances including monoamines, precursor amino acids and major metabolites from the same sample by a combination of HPLC-ECD and organic solvent extraction. This procedure may possibly be able to demonstrate a direct correlation between the biological disposition and biochemical effects of morphine in the brain. In the present study, therefore, the pharmacokinetics (disposition) and pharmacodynamics (biochemical effects on monoamine systems) of morphine in the mouse brain were investigated concurrently to determine whether biochemical changes are correlated with brain levels of the drug.

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MATERIALS AND METHODS

Reagents. Morphine HCl was obtained from Takeda Pharmaceuticals (Osaka, Japan) and dissolved in saline immediately prior to injection. Authentic standards for monoamine-related substances, including NA, DA, 5-HT, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxyphenylacetic acid [homovanillic acid (HVA)], 5-hydroxyindoleacetic acid (5-HIAA), tyrosine (Tyr) and tryptophan (Try) were all purchased from Sigma (St. Louis, MO). Internal standard substances, 3,4-dihydroxybenzylamine HBr (DHBA) (for amine assay) and 3,4-dihydroxyphenylpropionic acid (DOPPA) (for monoamine metabolite assay), were obtained from Aldrich (Milwaukee, WI) and ICN Pharmaceuticals (Plainview, NY), respectively. Reagent grade chemicals for extraction and chromatography were all obtained from a single source (Wako, Osaka, Japan) and used without further purification.

Animals. Male ICR mice, weighing about 25 g, were used. The animals were housed for 1 week in an air-conditioned room with a 12-hr light–dark cycle. Food and water were given *ad lib*. To eliminate the effects of diurnal variations all animals were killed between 10:00 a.m. and 12:00 noon.

In primary experiments, 10, 20 or 40 mg/kg morphine was injected intravenously and the mice were killed by microwave irradiation (5 kW, 0.6 sec), which prevented post mortem changes in monoamine-related substances, at 10 and 30 min, and 1, 2, 4, 8 and 24 hr after the injection. In the secondary experiments, 2.5, 5, 10, 20, 40 or 80 mg/kg morphine was injected intravenously and the mice were killed after 1 hr. Control animals received a saline injection and were killed 1 hr later. The brain was quickly removed and stored in a deep freeze (−80°) until the assays for morphine and monoamine-related substances.

Extraction of morphine and monoamine-related substances. Extraction of morphine, monoamines (NA, DA and 5-HT), precursor amino acids (Tyr and Try) and monoamine metabolites (DOPAC, HVA and 5-HIAA) was performed according to the procedures in previous reports [20, 21]. To the brain tissue was added 12 ml of *n*-butanol, 750 μl of 0.025 N HCl containing 400 ng/ml DHBA and DOPPA, and 100 μl of 0.1 M EDTA. The mixture was homogenized with a Polytron [20,000 rpm, 10 sec (Kinematika, Luzern, Switzerland)] in a glass-stoppered tube and 4 g of solid NaCl was added. The homogenate was shaken on a reciprocal shaker for 60 min and then centrifuged at 3000 rpm for 10 min. Ten millilitres of the butanol layer was transferred to another tube containing 200 μl of 0.1 N HCl and 20 ml of *n*-heptane. After shaking for 10 min, the tube was centrifuged at 3000 rpm for 5 min. The organic layer was transferred to a fresh tube and stored in a refrigerator at 4° until the monoamine metabolite assay. Twenty microlitres of the HCl layer was injected into the HPLC system. Two hundred microlitres of Tris–HCl buffer (pH 8.5) was added to the tube stored in the refrigerator. The tube was vortexed for 1 min and briefly centrifuged to separate organic and aqueous phases. Twenty microlitres of the aqueous phase was immediately injected into the HPLC system. The working standard for quantitative determination was treated exactly in the same way as the brain sample.

Determination of morphine and monoamine-related substances. The intracerebral level of morphine and monoamine-related substances were determined by HPLC-ECD according to the procedures in previous reports [20, 21]. All experiments were carried out using a liquid chromatographic system [Yanagimoto L-2000 (Kyoto, Japan)] with a six-port injector [Rheodyne 7120 (Berkeley, CA)] and a glassy carbon electrochemical detector (Yanagimoto VMD-101). The analytical column was an Ultraspere-ODS reversed-phase column [average particle size 5 μm, 250 × 4.6 mm i.d. (Altex, Berkeley, CA)]. The detector potentials were set at 725 and 600 mV vs an Ag/AgCl reference electrode for assay of morphine, monoamines and precursor amino acids, and of monoamine metabolites, respectively. Two different mobile phases were used for the complete determination. In the case of morphine, monoamines and precursor amino acids, a 0.1 M sodium citrate–citric acid buffer (pH 4.0) containing 1% tetrahydrofuran (THF) was used at a flow rate of 1.3 ml/min. On the other hand, a 0.075 M sodium citrate–citric acid buffer (pH 3.5) containing 1% THF, 10% methanol and 12% acetic acid was used at a flow rate of 0.8 ml/min for the monoamine metabolite assay. Both mobile phases were sonicated under vacuum in order to eliminate air bubbles which might interfere with the electrochemical detection. The content of each of the substances was determined by comparison of the relative peak heights against the internal standard in the sample and working standards. Working standards were run at regular intervals to check the sensitivity of the detector.

RESULTS

Elimination of morphine from the brain

Elimination curves for intracerebral morphine after intravenous injection of three different dosages are shown in Fig. 1. The brain levels of morphine were dependent on the dose injected, showing values of 705 ± 83 (mean ± S.D. from 10 determinations), 1274 ± 156 and 2769 ± 545 ng/g at 10 min after the injection of 10, 20 and 40 mg/kg morphine, respectively. Thereafter, the levels of morphine declined linearly and the biological half-life was estimated to be about 1 hr without difference among the dosages. The time course curves of the three doses were arranged in a parallel fashion. These data suggest that morphine passively penetrates into the brain from the blood, and is then eliminated. Only about 6% of the initial level (theoretical levels at 0 hr) remained in the brain for three dosages at 4 hr after the injection.

Effect of morphine on monoamine-related substances

No remarkable changes in the levels of steady-state monoamine transmitters (NA, DA and 5-HT) were observed in the brain after a single morphine injection in the dose range of 10–40 mg/kg. Morphine injections, however, caused dose-related increases in monoamine metabolite levels. Time courses for the monoamine metabolite levels (DOPAC, HVA

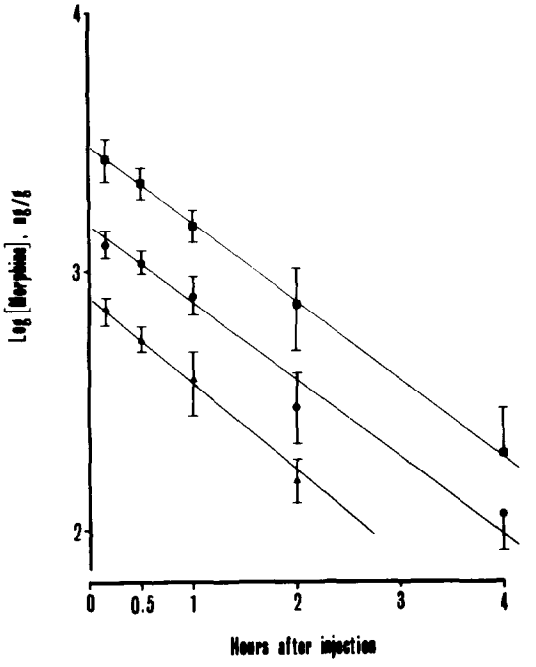

Fig. 1. Elimination curves of intracerebral morphine. Animals were injected intravenously with 10 (▲), 20 (●) and 40 mg/kg (■) doses of the drug and killed by microwave irradiation. Morphine was measured by high-performance liquid chromatography combined with electrochemical detection. The biological half-life was estimated to be about 1 hr at all three dosages. The points and vertical bars represent means ± S.D. from 10 determinations.

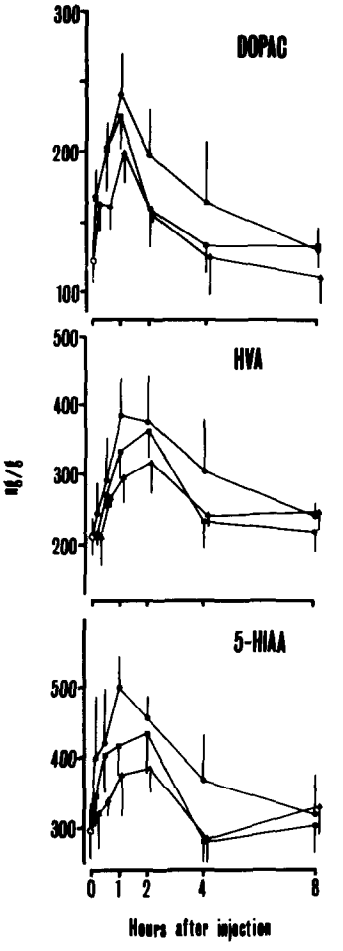

Fig. 2. Time courses of changes in intracerebral concns of monoamine metabolites after a single injection of morphine. Animals were injected intravenously with 10 (▲), 20 (●) and 40 mg/kg (■) doses of the drug. The brain was obtained after microwave irradiation and measured simultaneously for the metabolites using high-performance liquid chromatography combined with electrochemical detection. The points and vertical bars represent means ± S.D. from 10 determinations. The open circle shows a value for the control.

and 5-HIAA) in the brain after morphine injection are shown in Fig. 2. After injections of morphine (10–40 mg/kg), the brain levels of DOPAC were significantly increased with a peak effect occurring at 1 hr after the injection. Morphine (10–40 mg/kg) also caused significant increases in HVA and 5-HIAA levels. The maximal influences on HVA and 5-HIAA were delayed compared to that on DOPAC. At 4 hr after injection of different doses of morphine, the levels of monoamine metabolites returned to the control level except in cases where 40 mg/kg were injected. However, the incremental effect of morphine on these metabolites disappeared completely at 8 and 24 hr after the injection.

The brain concns of Tyr were decreased significantly to 70–50% of the control level at 2 or 4 hr after the injection of morphine (Fig. 3). Try was diminished slightly to about 90% of the control. These decreases were not closely related to the dose injected. The concns of these precursor amino acids also recovered completely to the control levels at 24 hr after the injection.

Correlation between morphine and monoamine metabolite levels in the brain

The maximal incremental effect of morphine on monoamine metabolites reached a plateau at an intracerebral concn of about 2000 ng/g. Higher levels of intracerebral morphine than this showed no further increase of monoamine metabolite levels. Thus, the effects of higher levels (greater than 2000 ng/g) of morphine were excluded in the calculation of correlation coefficients. The intracerebral levels of DOPAC and HVA were significantly correlated with the morphine level in the range from 50 to 2000 ng/g wet tissue. Further examinations were carried out on the relationships between the intracerebral concn of morphine and the ratios of metabolites to corresponding monoamine transmitters at 1 hr after injection of the drug (Figs 4–6). Significant correlations were noted between the morphine level and DOPAC/DA ($r = 0.7033$, $N = 64$, $P < 0.0001$) and HVA/DA ($r = 0.6455$, $N = 64$, $P < 0.0001$). On the other hand, the correlation between the morphine level and 5-HIAA/5-HT was lower ($r = 0.4699$, $N = 64$, $P < 0.0001$) than those for the dopaminergic system. The correlation coefficients were compared for the dopaminergic and serotonergic systems after conversion to a hyperbolic function. The coefficiency of DOPAC/DA and morphine

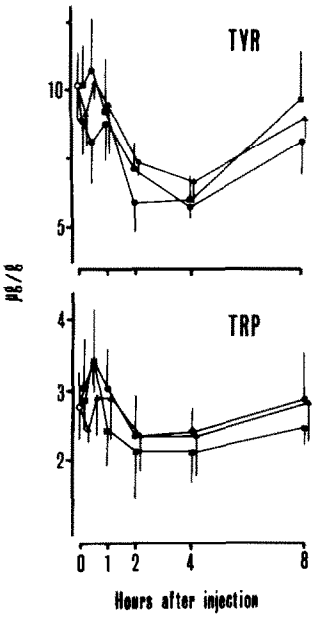

Fig. 3. Time courses of changes in intracerebral concns of tyrosine and tryptophan after a single injection of morphine at a dose of 10 (▲), 20 (●) and 40 mg/kg (■). The amino acids were measured by high-performance liquid chromatography combined with electrochemical detection. The points and vertical bars represent means ± S.D. from 10 determinations. The open circle shows a value for the control.

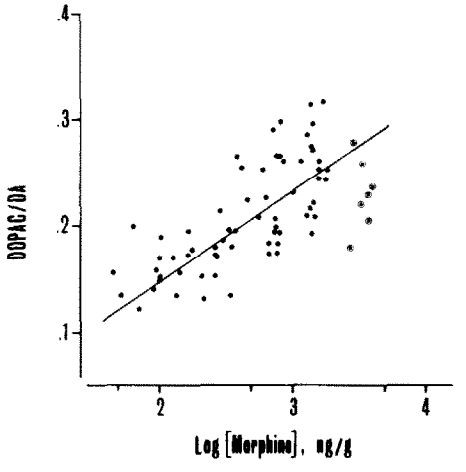

Fig. 4. Correlation between the ratio DOPAC/DA and the intracerebral concn of morphine. Animals were injected with morphine at different dosages ranging from 2.5 to 80 mg/kg. They were killed by microwave irradiation at 1 hr after the injection. The substances were measured simultaneously by high-performance liquid chromatography after butanol extraction. The regression curve was estimated at both primary and secondary degrees. The two resultant curves were parallel in the range of intracerebral concn from 70 to 1500 ng/g wet tissue. The linearity of the regression curve was restricted at a morphine concn of 2000 ng/g wet tissue. The maximal effect on monoamine metabolite levels was calculated from the regression curve of the secondary degree to be at 2345 ng/g wet tissue. The open circles with dot indicate points for concns of morphine higher than 2345 ng/g, for which the ratios were lower than the maximum. The regression coefficient for the linear curve was estimated to be 0.7033.

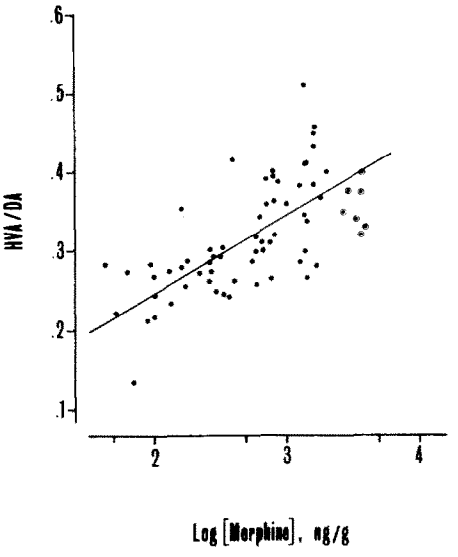

Fig. 5. Correlation between the ratio HVA/DA and the intracerebral concn of morphine. The regression coefficient for the linear curve was estimated to be 0.6455.

was significantly higher than that of 5-HIAA/5-HT and morphine ($t = 2.022$, 126 df, $P < 0.05$).

DISCUSSION

In the present study, the correlations between intracerebral levels of morphine (pharmacokinetics) and its biochemical effects on monoamine systems (pharmacodynamics) were investigated simultaneously using HPLC-ECD.

Attempts have been made to implicate the intracerebral monoamine concns in neurochemical mechanisms underlying the pharmacological actions of morphine. Morphine has been reported to affect the steady-state levels of monoamine transmitters [1,

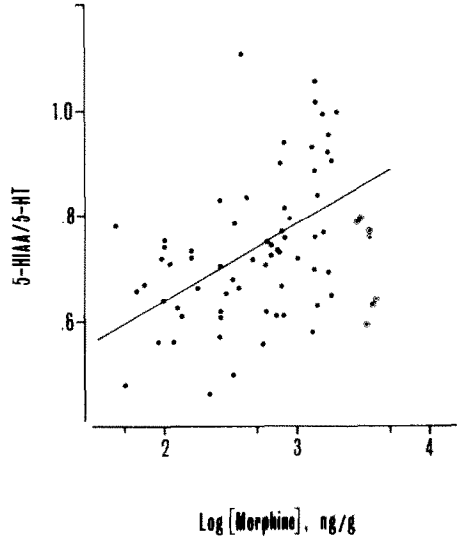

Fig. 6. Correlation between the ratio 5-HIAA/5-HT and the intracerebral concn of morphine. The regression coefficient for the linear curve was estimated to be 0.4699.

2], precursors [11, 12] and metabolites [4, 7–9, 11, 22]. The drug has also been shown to affect the turnover rates of these monoamine transmitters. However, the reported results seem to be conflicting. Such discrepant data may reflect mainly the routes, duration and doses of drug administration, and the differences in species or strains of animal. In particular, differences of route, duration or dose lead to different levels of the drug in the brain as well as the blood [23, 24], since the pharmacokinetics of the drug can be modified by such factors [25, 26]. It is important therefore to determine simultaneously the levels of morphine and monoamine-related substances in the same sample. The present method represents a relatively easy and sensitive procedure for the simultaneous determination of these substances.

The steady-state concns of monoamine transmitters were not markedly changed by a single injection of morphine. These results were not in agreement with previous reports [20, 27], in which decreases of the substances were observed. This could be due to the difference in killing procedures. In the present study, the animals were killed by microwave irradiation because a previous report had demonstrated that post mortem changes in transmitter substances occurred after decapitation [28]. On the other hand, morphine caused significant increases in the concns of the monoamine metabolites, DOPAC, HVA and 5-HIAA (Fig. 2). These changes were in agreement with previous findings indicating that morphine caused increases of the metabolites after a single injection [4, 7–9, 11, 12, 22]. Huang and Wajda [29] demonstrated that the increases in 5-HIAA and HVA in the brain induced by morphine were not due to inhibition of the elimination of these metabolites from the choroid plexus. These findings indicated that accumulation of the metabolites was caused by acceleration of the turnover rates of DA and 5-HT. This hypothesis is supported by the fact that the drug caused an increase in the incorporations of radiolabelled precursor amino acids into DA [3] and 5-HT [8, 10, 12], and an acceleration of the accumulations of HVA [9] and 5-HIAA [4, 8, 9] after probenecid treatment.

The principle purpose of the present study was to establish whether the intracerebral level of morphine and its biochemical effects on monoamine systems are quantitatively related or not. After morphine injection, direct correlations were noted between the concn of morphine and DOPAC or HVA, major metabolites of DA, in the brain. Also, the ratio DOPAC/DA or HVA/DA, which reflects the activity of DA metabolism, was correlated more significantly to the intracerebral morphine level than that 5-HIAA/5-HT. These results suggest that the morphine primarily affected the dopaminergic system in the brain. It has been suggested that the pharmacological action, analgesic effect, of morphine is closely related to an increased turnover of DA. Some investigators have observed a direct correlation between intracerebral morphine level and analgesic activity [13, 14]. These findings support the idea that the dopaminergic system is involved in the analgesic action of morphine.

The Tyr levels were observed to be decreased significantly, amounting to about 50–70% of the control levels, at 4 hr after morphine injection. This result seems unlikely to represent a direct action of the drug, because the intracerebral levels of morphine were lower at that time. The phenomenon could be a consequence of 'rebound phenomena' in the dopaminergic transmission, which are caused by increased synthesis of DA followed by rapid removal of morphine from the site of action [30].

In the present study, a direct correlation was observed between the intracerebral concn of morphine and monoamine transmitters. Correlations were also found for monoamine precursors and metabolites. These results indicate that the intracerebral monoaminergic systems, especially the dopaminergic system, are involved in the manifestation of the pharmacological effects of morphine. Furthermore, the present procedure can be said to represent a useful pharmacological approach for the simultaneous measurement of both pharmacokinetics and pharmacodynamics in the same sample, and constitute an important technique for use in pharmacological studies. The same approach may be applicable for drugs possessing phenolic hydroxy group(s) in their chemical structure.

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