PROTEIN SYNTHESIS IN MOUSE BRAIN DURING DEVELOPMENT OF ACUTE MORPHINE TOLERANCE

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Abstract—In order to observe the effects of morphine on protein metabolism in mouse brain, experimental procedures were carried out over a 7 hr period of infusion. When the analgesia reached a peak, namely around 2 hr after the start of infusion, the in vivo incorporation of radioactive leucine into protein estimated by the dual label technique was uniformly depressed in all the examined subcellular fractions of both brain and liver. After tolerance developed, however, the incorporation of leucine increased to a much higher level than the control in brain subcellular fractions and the increase was masked by naloxone. In contrast, the incorporation into a TCA soluble fraction of the brain S2, separated by Gray and Whittaker's method, was more than doubled even after 4 hr infusion. While the in vitro incorporation rate of the mitochondrial fraction significantly fluctuated during development of tolerance with naloxone that of the synaptosomal fraction did not fluctuate. The observed coincidence in the time course of development of tolerance and changes in the brain protein synthesis indicates a possible relationship between the phenomena, though the causal nature of the relationship could not be elucidated.

Morphine tolerance is a complex phenomenon and unfortunately few new clues have been presented. Recent reports (1-7) stated that the development of tolerance in rat or mouse could be aborted by inhibitors of the eukaryotic type of protein synthesis, suggesting that the opiate somehow induces abnormalities in the brain protein metabolism, which in turn leads to an ill-adapted state of the brain function, namely tolerance. The observations seem to be compatible with hypothetical mechanisms already proposed (8-11). A feasible next step would be to make an attempt to determine how the brain protein metabolism is modified by morphine.

In the present study, we examined the synthetic rate of brain protein change during development of acute tolerance in mice. To produce the tolerant animals, a continuous infusion method (12) was employed, since the method has proved to induce a measurable degree of tolerance within hours in this animal species and in addition assures no interference of the cyclic periods of narcosis and withdrawal in analysis of protein metabolism (13).

MATERIALS AND METHODS

Continuous infusion of morphine and measurement of analgesia

Mice (id strain male, weighing 20±1 g) were infused with morphine hydrochloride at a constant rate of 35 mg/kg/hr s.c. according to the method of Kaneto et al. (14) and
Analgesia was followed by the electric stimulation method (14). It was confirmed that the analgesic index reached a plateau within the first 2 hr of infusion and thereafter gradually waned, approaching that of the saline infused control group. The analgesic test was not applied to the animals used for the incorporation experiment in order to avoid the unwanted influence of the test procedure on the brain protein metabolism.

Incorporation of radioactive leucine into brain fractions in vivo

The dual label method (15) was employed. The experimental design involved two sets of animals, each containing two saline infused control mice and two morphine infused mice. In Set I, the control animals were injected with 1-leucine-4,5-3H (specific activity 800 mCi/m mole, 80 μCi/mouse) and the morphine infused with 1-leucine-U-14C (specific activity 100 mCi/m mole, 10 μCi/mouse), whereas in Set II the isotopes were given in the reverse order. At 1.5, 3.5 or 6.5 hr after start of infusion, the isotopes were injected i.p. and 1 hr later labelling was terminated by decapitation. Four brain samples from a pair of animals were combined and subjected to subcellular fractionation. For measurement of radioactivity, the sample was dissolved in 0.5 N NaOH to give a final concentration of 4 mg/ml as protein and an aliquot applied onto a glass fibre disc. The ratio of 14C (cpm) to 3H (cpm) in Set I and the reverse ratio in Set II were estimated and then the relative incorporation rate of radioactivity into a given fraction of the morphine infused group as compared to that of the saline infused group was calculated as described by Ch'ih and Devlin (15). Besides measuring the incorporation rate into the TCA insoluble fraction, the radioactivity present in the TCA soluble fraction of the S2 fraction was estimated after separation by ion exchange chromatography on a Dowex-50 x 8 column (1.8 × 10 cm). The TCA soluble fraction was diluted with water to five volumes and the pH adjusted to 3.0 with 1N NH4OH. The solution was applied on the column and the column was washed with 0.20 M ammonium formate buffer (pH 3.0). The eluate was discarded. The fraction eluted with 2.0 M ammonium acetate buffer (pH 7.0) was used to measure the radioactivity since leucine appears to this fraction. This fraction is hereafter referred to as the TCA soluble fraction.

Preparation of brain subcellular fraction and measurements of some enzyme activities

For separation of subcellular fractions, the animal was decapitated and the forebrain removed. Here, the forebrain included all tissue from rostral to the colliculus and cerebellum except for the olfactory bulb. The subsequent procedures were carried out at 0–4°C. The tissue was homogenized with five up and down strokes in twenty volumes of 0.32 M sucrose solution and the homogenate was separated into S2, the synaptosomal and the mitochondrial fractions according to the procedure of Gray and Whittaker (16). The homogeneity of the separated fractions was checked by measuring the following enzyme activities: succinic dehydrogenase (17), acetylcholinesterase (18), Na+, K+, and Mg2+-Ca2+-activated ATPase (19, 20). The protein was quantified by the method of Lowry et al. (21) using bovine serum albumin as the standard.

A typical distribution pattern of enzyme activities in the subcellular fractions is given in Fig. 1.
Preparation of liver supernatant fraction

Liver tissue was homogenized with twenty volumes of 0.25 M sucrose solution and the homogenate was centrifuged at 800 x g for 10 min. The supernatant was removed and centrifuged at 11,500 x g for 30 min.

Measurement of radioactivity incorporated into the hot TCA insoluble fraction by the glass fibre disc method (22, 23)

A given sample was dissolved in 0.5 N NaOH and an aliquot, usually 50 µl, placed onto a glass fibre disc (Whatman GF/A, 2.1 cm in diameter). The disc was air-dried and dipped into a glass beaker containing ice cold 10% TCA solution. After standing at 4°C overnight and washing with 5% TCA, the disc was placed in a fresh 5% TCA and heated at 90°C for 30 min. The disc was washed with 5% TCA, warm ethanol, ethanol-ether mixture and warm ether, in that order, and, after air drying, transferred into a vial containing 5 ml of toluene scintillator (PPO 0.4%, POPOP 0.01% in toluene). The radioactivity on the disc was measured in a liquid scintillation counter with counting efficiency of 82% for 14C alone or 53 and 29% for 14C and 3H simultaneously measured, respectively.

Incorporation of radioactive leucine into brain fractions in vitro

Both brains from a pair of morphine and saline infused animals, were separately processed to obtain the corresponding synaptosomal and mitochondrial fractions as described above. The fraction containing 0.70 mg as protein was suspended in 1.0 ml of a medium containing 33 mM of Tris-Cl (pH 7.4), 100 mM NaCl, 20 mM KCl, 100 mM sucrose and 1.0 µCi of L-leucine-U-14C (216 mCi/mmole). Incubation was carried out in a bath shaker at 37°C with mild agitation (60 strokes/min) for 30 min and stopped by adding one volume of ice cold 10% TCA containing 6 mM of nonradioactive leucine. In order to minimize bacterial contamination and its reflection in the incorporation rate, the following precautions were taken; the blank in which the reaction mixture received the tissue fraction just prior to the addition of TCA, was prepared and the radioactivity of the blank was subtracted from that of the sample. All the reagents and apparatus used before the addition of TCA, were autoclaved. The TCA treated mixture was stored at 4°C overnight. The formed precipitate was collected by centrifugation, dissolved in 0.5 ml of 0.5 N NaOH and reprecipitated with 1.5 ml of 10% TCA. The washed precipitate was collected by

| Fraction | Activity/µg protein/30 min |
|----------|---------------------------|
| S.D.H.   | 0.8 M                     |
|          | 1.2 M                     |

Fig. 1. Distribution of enzymes among the myelin, the synaptosomal and the mitochondrial fractions (from the top to the bottom of discontinuous density gradient)
centrifugation and dissolved in 0.30 ml of 0.5 N NaOH. The aliquot was subjected to protein determination and radioactivity measurement.

Drugs used include: morphine hydrochloride (Takeda), cycloheximide (Tanabe), chloramphenicol (Sigma). Naloxone was a gift from Sankyo Co., Ltd. Radioactive leucine was obtained from Daiichi Chemicals.

RESULTS

Effect of morphine infusion on the in vivo incorporation rate of radioactive leucine into the subcellular fractions

Using the double labelling technique, the relative incorporation rate of leucine in the morphine infused group to that in the saline infused group was estimated for the following periods; 1.5 to 2.5 hr after start of infusion when analgesia reached the plateau, 3.5 to 4.5 and 6.5 to 7.5 hr corresponding to the periods in which tolerance was established and possibly reinforced, respectively (Fig. 2). For the first period, the rate of the brain fractions of the morphine infused group uniformly slowed down. When morphine infusion was extended to 4.5 hr, however, all the fractions exhibited an increase in the rate. In the supernatant fraction of liver, the rate was initially decreased by morphine infusion and later returned to the control level.

Naloxone is known to completely mask morphine analgesia if the antagonist is infused concurrently with morphine at a dose ratio of more than one tenth the morphine dose. This agent is also used to abort development of either tolerance or dependence insofar as examined for the ensuing several days after infusion (24). When the mice were treated under the same condition, there was the initial decrease in the labelling rate as observed in the group infused with morphine alone (Fig. 3). On further continuation of the concomitant infusion, however, the incorporation rate returned to and did not exceed the control level.

Fig. 2. Effects of morphine infusion on the in vivo incorporation of radioactive leucine into the proteins of the brain subcellular fractions ○○○○: the S2 fraction, ●●●●: the synaptosomal, ××××: the mitochondrial, and ▽▽▽▽: the supernatant of liver.

Fig. 3. Effects of morphine plus naloxone infusion on the in vivo incorporation of radioactive leucine into the proteins of the brain subcellular fractions ○○○○: the S2 fraction, ●●●●: the synaptosomal, ××××: the mitochondrial, and ▽▽▽▽: the supernatant of liver.
FIG. 4. Effects of morphine infusion on the in vivo incorporation of radioactive leucine into the S₂-TCA soluble fractions of brain.

On the other hand, the incorporation rate of radioactivity into the TCA soluble fraction was highly increased by morphine infusion. The 2 hr infusion more than doubled the rate and such increase was still observed after 4 hr infusion (Fig. 4).

Effects of morphine infusion on the in vitro incorporation rate of radioactive leucine into the mitochondrial and synaptosomal fractions

When each pair of the synaptosomal fractions obtained from the morphine and saline infused animals for 2, 4 or 7 hr were compared, it was observed that morphine infusion had no significant influence on the rate (Fig. 5). In contrast, the incorporation rate measured with the mitochondrial fraction significantly changed depending on the duration of the morphine infusion (Fig. 5). The fraction from the 2 hr morphine infused group

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FIG. 5. Effects of morphine infusion on the in vitro incorporation of radioactive leucine into the proteins of the brain synaptosomal and mitochondrial fractions

- - - - - - - - - - : the synaptosomal fraction,
×××××××××× : the mitochondrial fraction.

Each value represents the mean of 5 experiments and the vertical bar S.E. The value of 2 hr of the mitochondrial fraction was significantly different from that of 4 hr of the same fraction (P<0.05).

FIG. 6. Effects of morphine plus naloxone infusion on the in vitro incorporation of leucine into the proteins of the brain synaptosomal and mitochondrial fractions.

- - - - - - - - - - : the synaptosomal fraction,
×××××××××× : the mitochondrial fraction.
exhibited an increased rate of incorporation as compared with the 4 hr infused group in which the rate fell to about 80% of the saline infused control. Such a biphasic change failed to appear in the fraction when naloxone was infused concurrently with morphine (Fig. 6).

Effects of cycloheximide and chloramphenicol on the in vitro incorporation rate of radioactive leucine into the mitochondrial and synaptosomal fractions

The synaptosomal fraction was more sensitive to chloramphenicol than to cycloheximide, whereas the reverse results were obtained with the mitochondrial fraction (Table 1).

| Table 1. Effects of two protein synthesis inhibitors on the in vitro incorporation of radioactive leucine into the protein of the synaptosomal and mitochondrial fractions |
|-------------------------------------------------|-----------------|-----------------
| Amount added | Synaptosome | Mitochondria |
| none (control) | 141 (100) | 472 (100) |
| cycloheximide | 10 µg | 108 (77) | 170 (36) |
| | 100 | 92 (65) | 141 (30) |
| chloramphenicol | 10 | 72 (50) | 406 (86) |
| | 100 | 50 (35) | 354 (75) |

DISCUSSION

During the continuous infusion of morphine, the incorporation rate of radioactivity of leucine into the proteins of any examined subcellular fractions of brain changed in a biphasic manner. The rate was first depressed and, after establishment of analgesic tolerance, it reached a level higher than in the control group. Appearance of this increased phase was masked by the concomitant infusion of naloxone with morphine. The coincidence in time course of development of tolerance and appearance of the second phase suggests a possible relationship of tolerance with the turning point of the protein synthesis.

On the other hand, neither the biphasic change nor masking by naloxone of the incorporation of radioactive leucine could be obtained in the liver supernatant fraction thus indicating the difference between subcellular fractions from different organs.

In the present study, we assumed that the synaptosomal fraction would mainly reflect the protein synthesis of the nerve cell and the mitochondrial that of both the nerve and glia cells and also that 1 hr labelling time would minimize the extent of contribution of the axoplasmic flow in the synthetic rate of protein. The obtained results indicated, however, that both fractions exhibited similar patterns of changes in the synthetic rate.

Recently, Kushinsky reported that a single dose of morphine (100 mg/kg) to mice inhibited the in vivo incorporation rate of radioactive leucine into the synaptosomal fraction but not that into the mitochondrial (25). The discrepancy may be partly attributed to the difference in labelling time, that is 4 hr in his experiment and 1 hr in the present work. In order to estimate the synthetic rate of protein more correctly, other factors should be taken into account, particularly the availability of the precursors. As shown in Fig. 3, it was indeed observed that the 2 hr morphine infused group retained 2.5 times
higher radioactivity in the TCA soluble fraction than the control, and after 4 hr infusion, the incorporation was substantially depressed. Previously, Clouet and Ratner have observed that intracisternally injected radioactive leucine was longer retained by the brain tissue of the morphine treated rat than that of the control and the conclusion was that such effect of the drug is due to inhibition of efflux of amino acids from the brain tissue (26–29).

Recently, it has been demonstrated that the synaptosomal membrane itself possesses the ability to synthesize some protein species (30–32) and some of mitochondrial proteins are synthesized in the prokaryotic mitochondrial ribosomes (33–38). By morphine infusion, the in vitro protein synthesis of the synaptosomal fraction was not significantly affected but that of the mitochondrial changed depending on the infusion time. Such change was masked by naloxone. On the other hand, addition of morphine to the incubation mixture had no effect on the protein synthesis in either fraction. These results are not in parallel with the observations of Cox and Osman (6). They reported that chloramphenicol, a specific inhibitor of protein synthesis on the prokaryotic type of ribosome, failed to abort development of analgesic tolerance in the rat on morphine infusion. If the observed changes in the mitochondrial fraction would be in any way related to the development of tolerance, such changes might have occurred in the eukaryotic type of protein synthesizing machinery which exists in the mitochondrial fraction prepared by the conventional method, possibly as the contaminant (33, 35, 38). As shown in Fig. 1, the enzyme study proved a good separation of two granular fractions, and chloramphenicol and cycloheximide behaved differently on the protein synthesis of the fractions (Table 1) as has also been observed by other investigators (39–43). Thus it would be more adequate to conclude that the morphine infusion specifically changes the protein synthesis which occurs in the fraction separated as the mitochondrial. Further attempts to clarify whether or not such changes would be specific to the synthesis of certain protein molecules, however, failed mainly due to the low radioactivity incorporated into the fraction (unpublished observations).

The experimental results reported herein indicate that morphine infusion induces significant changes in the brain protein synthesis, some of which appear to be related to development of morphine tolerance, and in addition provide the basic aspects of morphine action which are useful for examining the effects of morphine on the brain protein synthesis in a more detailed manner. Such experiments along these lines have been reported by other investigators (44–46) as well as from our own laboratory (47).

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