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PHARMACOLOGICAL STUDIES OF ANALGESICS—VI.
THE ADMINISTRATION OF MORPHINE AND CHANGES IN ACETYLCHOLINE METABOLISM MOUSE BRAIN

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Abstract—Mice were treated with increasing doses of morphine for 6 weeks and changes in brain acetylcholine (ACh) metabolism were investigated.

The administration of a single dose of morphine (100 mg/kg) caused a marked increase in brain ACh content in normal animals, but this increase was not found in animals that had received chronic administration of morphine.

Morphine did not affect the activity of cholinesterase (ChE) *in vivo*, and no significant difference was observed between the brain ChE activity of normal and morphinized animals.

These results indicate that ACh metabolism in brain may be involved in the mechanism of the development of tolerance to morphine.

OF THE numerous explanations for the mechanism of the development of tolerance to, and physical dependence on, morphine, it is widely accepted that alterations in the physiological function of the central nervous system may play a part in these phenomena, but there is insufficient evidence to prove this hypothesis.

Because of the signs of hyperactivity of the autonomic nervous system during morphine abstinence in man and monkey, and the fact that morphine has a mild anticholinesterase activity and produces many symptoms suggestive of parasympathetic stimulation in the intact animal, it seemed of interest to investigate acetylcholine (ACh) metabolism in brain during the development of tolerance to morphine and after withdrawal from the drug.

In the present study we focused our attention on the possibility that the changes of ACh metabolism in brain are involved in the physiological adaptation that constitutes tolerance to and/or physical dependence on morphine.

METHOD

Animals. The experiments were done with male albino mice of the ddO strain. Their body weight at the beginning of experiments was 13–15 g. Mice were divided into groups of five animals and housed in wire-bottomed cages. They were fed stock diet and water *ad libitum*.

Administration of drugs. The mice were given morphine hydrochloride, starting with a dose of 20 mg/kg and gradually increasing it (twice daily, 7 days a week), over a period of 6 weeks, to 120 mg/kg.

Extraction of brain ACh was performed by the method of Richter and Crossland.[1] Animals were sacrificed by immersion in acetone–dry ice and the brains were removed. The weighed brains were broken up with a glass rod at 0° and suspended in a mixture of 1 ml of 0·14 N acetate buffer and 3 ml eserinized acidified amphibian Ringer-Locke solution.

The Ringer-Locke solution before acidification contained 6·5 g NaCl, 0·14 g KCl, 0·2364 g $CaCl_2 \cdot 6H_2O$, and 0·2 g $NaHCO_3$ per liter. It was brought to pH 4·0 with 0·22 N HCl, and eserine sulfate (1 : 4000) was added.

The mixture was quickly placed in a boiling water bath, allowed to cool, stirred with a glass rod, and centrifuged. After removal of the supernatant solution by decanting, the residue was washed with eserinized amphibian Ringer-Locke solution (pH 4·0) and the combined supernatant solution was kept at 4° until assayed. Before analysis, the extract was neutralized to pH 7·2 with 0·1 N NaOH.

ACh was estimated by measuring the contraction of the eserinized frog rectus abdominis muscle preparation.[2]

Estimation of cholinesterase activity in mouse brain. After decapitation, the cerebral hemispheres were dissected out, weighed, and homogenized with an appropriate volume of Krebs-Ringer bicarbonate buffer (pH 7·4) in a Potter homogenizer at 0–5°. Homogenates were then diluted with the same buffer to give a final concentration of 0·5% (w/v).

Cholinesterase activity was measured manometrically by Ammon's method[3] and the conventional Warburg technique at 37·5° with 95% N_2 and 5% CO_2 as a gas phase. Final concentration of 2×10^{-3} M of ACh chloride was used as substrate.

In the chronic experiments, mice were sacrificed 18 hr after the final dose of drugs.

RESULTS

ACh Content in whole brain

Normal mice. The administration of a single dose of morphine (100 or 200 mg/kg) caused a marked increase in the cerebral content of ACh 30 min after subcutaneous injection of the drug, although 20 mg/kg failed to bring about such an effect (Table 1).

TABLE 1. ACh CONTENT IN MOUSE BRAIN 30 MIN AFTER THE ADMINISTRATION OF A SINGLE DOSE OF MORPHINE-HCl

Dose, mg/kg	No. of experiments	ACh, μg/g	Increase, %
0	6	1·64 ± 0·05	0
20	5	1·65 ± 0·08	0·6
100	6	1·93 ± 0·05	17·7*
200	5	2·17 ± 0·12	32·3*

* Significant ($P < 0·01$).

The maximal increase in brain ACh was obtained at 30 min after the injection and declined a little but not further within 60 min (Fig. 1). Therefore, in the following experiments, animals were sacrificed 30 min after drug administration.

Chronic administration of morphine. The estimations of brain ACh content were performed weekly during chronic administration of morphine and at days 2, 4, 6, 10, and 15 after withdrawal from the drug.

As shown in Fig. 2, the average brain content of ACh was not changed from that of control animals by chronic treatment with morphine or by withdrawal from the drug, when measured 15 hr after the previous injection. The ACh content in the brains of these animals was increased in parallel with the increase of their body weight

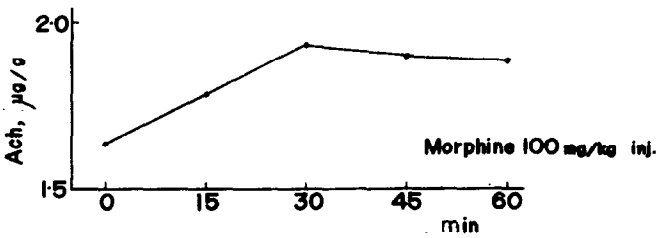

FIG. 1. ACh content in mouse brain after the administration of a single dose of 100 mg morphine-HCl/kg.

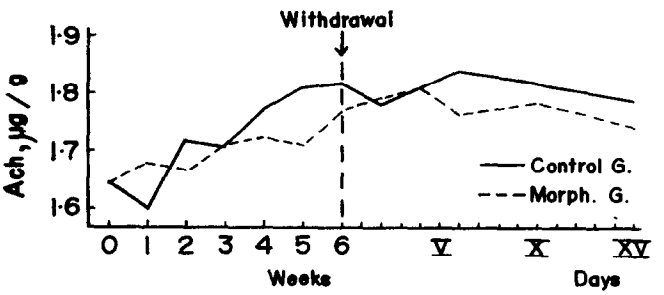

FIG. 2. Changes of brain ACh content in morphinized mice.

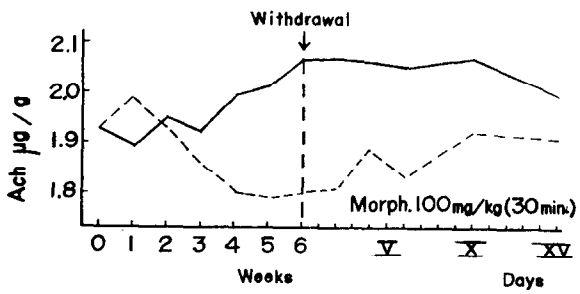

FIG. 3. Changes of brain ACh content in morphinized mice 30 min after the administration of 100 mg morphine-HCl/kg.

An increase of the brain ACh content induced by the additional single dose of 100 mg morphine/kg 15 hr after the scheduled injection was gradually minimized with the chronic administration of morphine. The same dose of morphine no longer produced an increase in brain ACh content in animals that had received morphine for 4 to 6 weeks. After withdrawal from morphine, the same dose of morphine gradually began to increase the content of brain ACh to the previous level (Fig. 3).

Fig. 4 shows the daily changes in the percentage increase of brain ACh induced by a single dose of 100 mg morphine/kg in morphinized mice as compared with the control animals, which had received saline solution (Fig. 4). Morphine exerted a similar action on rats.*

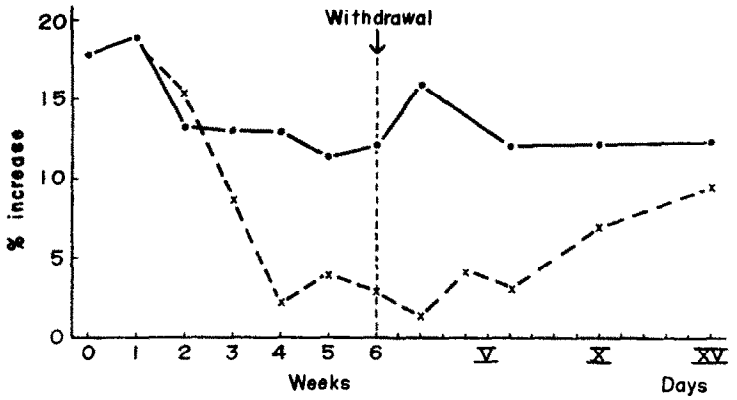

FIG. 4. Daily changes of the percentage increase of brain ACh content induced by a single dose of 100 mg morphine-HCl/kg in normal and morphinized mice.

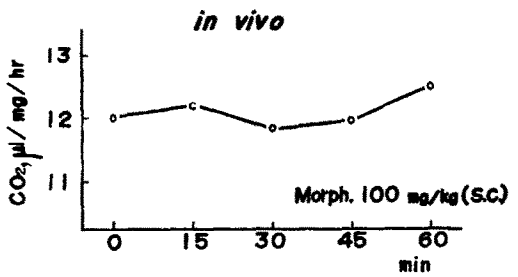

FIG. 5. Effect of the administration of a single dose of 100 mg morphine-HCl/kg on brain ChE activity in normal mice.

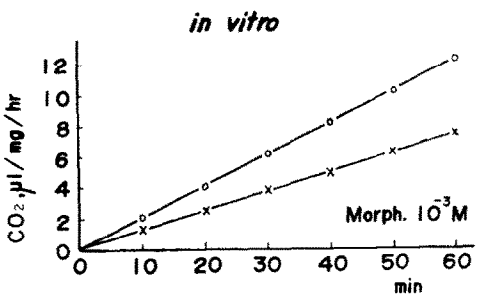

FIG. 6. Effect of morphine on brain ChE activity in normal mice.

ChE activity in mouse brain

Normal mice. A single dose of 20 mg or 100 mg morphine/kg did not affect the activity of brain ChE *in vivo* (Fig. 5). In experiments *in vitro*, on the other hand, addition of 1×10^{-3} M morphine inhibited the ChE activity of mice brain about 40 per cent (Fig. 6).

* Unpublished data.

Chronic administration of morphine. No significant difference was found between the activity of brain ChE in chronically treated mice and control animals throughout the experimental period (Fig. 7). The inhibitory effect of morphine on the brain ChE activity *in vitro* did not change either during morphinization or after the withdrawal from the drug (Fig. 8).

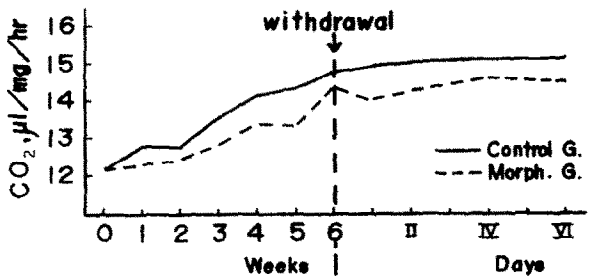

FIG. 7. Changes of brain ChE activity in control and morphinized mice.

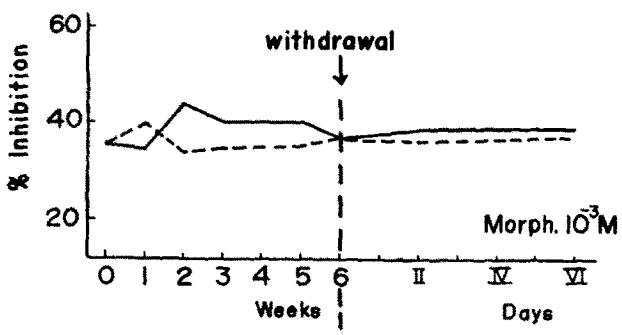

FIG. 8. Changes of the inhibitory effect of morphine on brain ChE activity in normal and morphinized mice *in vitro*.

DISCUSSION

Since Bernheim and Bernheim[4] demonstrated the inhibitory effect of morphine on ChE *in vitro*, there have been several reports[5–7] that cholinergic signs after morphine administration may be the result of the accumulation of ACh in brain by inhibition of ChE.

This hypothesis is indeed of interest in view of the fact that some anticholinesterase agents act synergistically with morphine in producing analgesia,[8] and that many of the manifestations of morphine action suggest that a cholinergic mechanism may be involved in the effect of morphine on the central nervous system.[9–12]

On the other hand, Takagi[13] and Young *et al.*[14] failed to show the inhibitory effect of morphine on brain ChE activity *in vivo* and concluded that ACh in brain has no relationship to morphine analgesia.

In our present study we also found that the activity of brain ChE was not affected by the administration of rather large doses of morphine, and in addition the ACh content or ChE activity in brain showed no difference between normal and morphinized animals.

These observations may support the concept that brain ACh may not be directly related to the analgesic effect of morphine. However, if the changes of brain ACh content are a factor in the development of physical dependence, the present experimental evidence on ACh content and ChE activity may correspond with the results of our previous experiments[15] in which we failed to demonstrate physical dependence in morphinized mice.

Several investigators have tried to explain the mechanism of development of tolerance to morphine by established differences between enzyme activities in tolerant and nontolerant organisms. For example, less N-demethylating capacity and less transferase activity were shown in livers obtained from tolerant rats than in those from nontolerant rats;[16–18] but we could not find this difference for brain ChE.

On the other hand, as shown in the above experiments with normal mice, a single dose of morphine (100 mg/kg) caused an increase in brain ACh level 30 min after administration. If this effect reflects the central depressive action of morphine, it could be expected that the development of tolerance might be related to changes in ACh content produced by morphine.

Actually, the increase of brain ACh content after morphine injection was reduced in morphinized animals, and it could not be observed after 3–4 weeks' treatment with morphine. After withdrawal, the capacity of morphine to increase ACh was restored gradually to the control level. These changes in morphine action seem to parallel the development of tolerance to its analgesic or sedative effect, which was described in our previous paper.[15]

We cannot explain the meaning and the mechanism of the inhibitory effect of morphine at the present time, but since it is not probable that morphine accelerates ACh synthesis in mammalian brain,[19, 20] the increase of ACh content that follows a large dose of morphine appears not to be the result of the inhibition of ChE or the acceleration of ACh synthesis in brain, but the result of decreased liberation of ACh from its storage site.

Indeed, it has been demonstrated that morphine inhibits the release of ACh from the isolated guinea pig intestine,[21] from the electrically stimulated guinea pig ileum,[22] and from the preganglionic element of the superior cervical ganglion;[23] and in cell-free experiments Torda and Wolff[24] and Kumagai and Ebashi[25] found no significant effect on the formation of ACh.

Richter and Crossland[1, 26] and Elliott *et al.*[27] have shown that sedatives and anesthetics induce elevations of brain ACh, primarily in the "bound" form, presumably by interfering with its normal release from bound to 'free'.

In unpublished work we have obtained additional support for this hypothesis in showing that the KCl-stimulated liberation of ACh from brain slice was inhibited by the addition of 1×10^{-3} M morphine.[19] However, in these experiments the medium contained a high concentration of eserine in order to protect the hydrolysis of liberated ACh from ChE, and we are working on further experiments under physiological conditions.

REFERENCES

1. D. Richter and J. Crossland, *Amer. J. Physiol.* **159**, 247 (1949).
2. H. C. Chang and J. H. Gaddum, *J. Physiol. (Lond.)* **79**, 255 (1933).
3. R. Ammon, *Pflügers Arch. ges. Physiol.* **233**, 486 (1933).
4. F. Bernheim and M. L. C. Bernheim, *J. Pharmacol. exp. Ther.* **57**, 427 (1936).
5. D. Slaughter, J. C. Parson and H. D. Munal, *J. Amer. med. Ass.* **115**, 2058 (1940).
6. D. Slaughter and E. G. Gross, *J. Pharmacol. exp. Ther.* **68**, 96 (1940).
7. D. Slaughter and D. W. Munsell, *J. Pharmacol. exp. Ther.* **68**, 104 (1940).
8. W. Schaumann, *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **237**, 229 (1959).
9. C. K. Himmelsbach, *J. Pharmacol. exp. Ther.* **73**, 91 (1941).
10. N. Emmelin and R. Strömblad, *Acta physiol. scand.* **24**, 261 (1951).
11. P. A. Nasmyth, *Brit. J. Pharmacol.* **9**, 95 (1954).
12. E. L. Way, *J. Pharmacol. exp. Ther.* **114**, 43 (1955).
13. H. Takagi, *Folia pharmacol. jap.* **48**, 300 (1952).
14. D. C. Young, R. A. Ploeg, R. M. Featherstone and E. G. Gross, *J. Pharmacol. exp. Ther.* **114**, 33 (1955).
15. K. Hano, H. Kaneto and T. Kakunaga, *Jap. J. Pharmacol.* **13**, 214 (1963).
16. J. Axelrod, *Science* **124**, 263 (1956).
17. J. Axelrod and J. Cochin, *J. Pharmacol. exp. Ther.* **121**, 107 (1957).
18. J. Axelrod and J. Cochin, *J. Pharmacol. exp. Ther.* **125**, 105 (1959).
19. K. Hano, H. Kaneto, T. Kakunaga and N. Moribayashi. Unpublished data.
20. J. H. Quastel and W. J. Johnson, *Nature (Lond.)* **171**, 602 (1953).
21. W. Schaumann, *Brit. J. Pharmacol.* **12**, 115 (1957).
22. W. D. Paton, *Brit. J. Pharmacol.* **12**, 119 (1957).
23. E. W. Pelikan, *Ann. N.Y. Acad. Sci.* **90**, 52 (1960).
24. C. Torda and H. G. Wolff, *Amer. J. Physiol.* **151**, 345 (1954).
25. H. Kumagai and S. Ebashi, *Jap. J. Pharmacol.* **4**, 24 (1954).
26. J. Crossland, *J. Ment. Sci.* **99**, 247 (1953).
27. K. A. C. Elliott, R. L. Swank and N. Henderson, *Amer. J. Physiol.* **162**, 469 (1950).