Antagonism of Morphine Analgesia by Adenine, Adenosine, and Adenine Nucleotides[1] (37680)

D. R. H. GOURLEY[2] AND SUZANNE K. BECKNER

Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22903

Ho, Loh and Way have reported (1, 2) that the administration of cyclic adenosine 3′,5′-monophosphate (cAMP) to naive mice or rats antagonized the analgesic effect of morphine when tested by the tail-flick response to a thermal stimulus. The antagonism of morphine analgesia appeared to be relatively specific for cAMP because dibutyryl cAMP, a phosphodiesterase-resistant cAMP analog, and theophylline, a phosphodiesterase inhibitor which reduces the breakdown of cAMP, acted in a similar manner whereas uridine, guanosine, or cyclic cytidine 3′,5′-monophosphate did not (unpublished results cited by Ho *et al.*, Ref. 3). Surprisingly, cAMP was just as effective in antagonizing morphine analgesia when it was administered iv as it was when administered intracerebrally although the maximum antagonism after iv administration required 6 hr to develop. Attempts to demonstrate that cAMP can traverse cell membranes have been unsuccessful (4, 5). Furthermore, it is unlikely that a significant amount of exogenous cAMP would remain in the body 6 hr after injection. It seems possible that soon after iv injection cAMP was transformed in the body to other products which may have penetrated to the site of morphine's analgesic action. The present experiments were performed to delineate further the specificity of cAMP as an antagonist of morphine analgesia by testing certain other purines as well as some compounds whose concentration in the body might be expected to increase as a result of cAMP administration. Because of the potential importance of establishing a relationship between cAMP levels and morphine analgesia, a second objective of this work was to confirm the effect of cAMP reported by Ho *et al.* (1, 2) by using analgesic assays different from the tail-flick technique.

Materials and Methods. Male Swiss-Webster mice (26–33 g), the strain used in the original work reported by Ho *et al.* (1), were obtained from Hilltop Lab Animals, Inc. (Scottdale, Pennsylvania) and maintained in the laboratory at 21–23° for at least one day, but no longer than 3 days, before use. Food (pellets of Purina mouse chow) and water were supplied *ad libitum*. Each mouse was used only once. Assays were performed in the morning in a room relatively free of extraneous noise and activity.

Cytidine, uridine, adenosine, adenosine 5′-monophosphate·$2H_2O$ (AMP), and disodium adenosine 5′-triphosphate (ATP) were purchased from P-L Biochemicals, Inc. (Milwaukee, Wisconsin); disodium adenosine 5′-diphosphate (ADP), cAMP, 2′-deoxyadenosine, and 8-bromoadenosine from Sigma Chemical Co. (St. Louis, Missouri); adenine and hypoxanthine from Schwarz/Mann (Orangeburg, New York); phenyl-*p*-benzoquinone [2-phenyl-1,4-benzoquinone, (PBQ)] from Eastman Organic Chemicals (Rochester, New York); morphine sulfate from Merck and Co., Inc. (Rahway, New Jersey). The purines, nucleosides, and nucleotides were prepared in 0.9% sodium chloride and adjusted, when necessary, to the pH of the control 0.9% sodium chloride solution. These solutions were used to pretreat the animals 18–

[1] This investigation was supported in part by NSF Grant GB 29558. The technical assistance of Cheryl K. Cooper in the preliminary experiments and the helpful suggestions of Dr. Gary Brooker and Prof. W. Schneider are gratefully acknowledged.

[2] Present address: Department of Pharmacology, Eastern Virginia Medical School, 358 Mowbray Arch, Norfolk, Va. 23507.

19 hr before the analgesia tests except in a few preliminary experiments in which the interval was 6 hr. The operator did not know which of the two solutions in a given pretreatment contained the test chemical. For the hot-plate test, morphine was prepared in 0.9% sodium chloride; for the stretching test, morphine was prepared in distilled water. Morphine solutions were prepared at concentrations such that each dose was given in a volume of 10 μl/g. ED_{50} values are expressed as milligrams of morphine base per kilogram of mouse body weight.

Hot-plate test. The apparatus and method used were essentially the same as described by Eddy and Leimbach (6). The closed copper vessel contained boiling acetone (56.5°). A plexiglass cylinder (15 cm diam, 25 cm high), open on both ends, was placed on top of the copper vessel to confine the animal to the constant temperature plate. A mouse was placed within the cylinder and the elapsed time for the reaction endpoint noted in seconds. The most reliable endpoint was considered to be licking the forepaws or hindpaws which occurred in mice not treated with morphine with a mean latency of about 10 sec. A mouse was removed as soon as this reaction endpoint was observed, or in 30 sec if it failed to react. To test the effect of pretreatment on morphine analgesia, a 6-point assay was performed with three dosage levels of morphine in each assay. Each dose was administered to a group of 8 mice; 3 groups were pretreated with test chemical and 3 groups were pretreated with the control saline solution. In a preliminary experiment, maximum analgesia to the thermal stimulus occurred 30 min after injection sc of 10 mg/kg morphine. Each mouse was tested, therefore, exactly 30 min after morphine administration. The percentage of mice in each group not reacting within 30 sec was plotted on probability paper against the dose of morphine.

Stretching test. The method used was similar to that described by Smits and Takemori (7). PBQ, used as the irritant to induce stretching, was prepared fresh daily by dissolving 10 mg in 2.6 ml 95% ethanol and diluting to 50 ml with warm water (final concentration, 0.02% PBQ in 5% ethanol). The PBQ solution was stored in a darkened bottle during use. Each mouse received 2 mg/kg PBQ ip. In a preliminary experiment, the maximum number of stretches occurred 7–13 min after administration of PBQ. Thus, morphine or water (control) was injected at 0 time, PBQ was injected ip into all animals at 20 min, the counting of stretches commenced at 27 min and stopped at 33 min. The midpoint of the 6-min observation period was at 30 min after administration of morphine and 9 min after administration of PBQ. Control mice (N = 130) stretched an average of 20 times during this observation period in response to PBQ. The number of stretches was reduced by morphine. To test the effect of pretreatment on this morphine action, an 8-point assay was performed. Four dosage levels of morphine were administered to groups of control animals and to groups of animals pretreated with a test chemical. Each group consisted of 10 mice. After injection of PBQ, each mouse was placed in an individual transparent container. Five mice were observed at one time. From the control group which had received water, the percent protection against PBQ-induced stretching response was calculated as follows:

$$\% \text{ protection against PBQ-induced stretching responses} = 100 - \left(\frac{S_x}{S_c} 100 \right)$$

where S_x = total number of stretching responses per 10 mice in the test group and S_c = total number of stretching responses per 10 mice in the control group. The percent protection in each group was plotted on probability paper against the dose of morphine.

For both types of test, the ED_{50} of morphine and its 95% confidence limits were calculated by the method of Litchfield and Wilcoxon (8).

Results and Discussion. In demonstrating that cAMP antagonized the analgesic effect of morphine, Ho *et al.* (1, 2) used the tail-flick response to a thermal stimulus. It seemed desirable to confirm the analgesia antagonism action of cAMP by procedures

TABLE I. The Effect of Pretreatment with Various Purines, Nucleosides, or Nucleotides on the ED_{50} of Morphine in Mice as Determined by the Hot-Plate Test and the Stretching Test.

	Hot-plate test			Stretching test		
	ED_{50} (mg/kg)			ED_{50} (mg/kg)		
Compound[a]	Control	Pretreated	Potency ratio	Control	Pretreated	Potency ratio
cAMP	14.3 (10.7–19.2)	23.9 (17.3–33.0)	1.67 (1.08–2.59)[b]	0.40 (0.25–0.63)	0.87 (0.59–1.29)	2.18 (1.19– 3.99)[b]
	11.8 (8.6–16.3)	20.0 (13.6–29.4)	1.69 (1.02–2.79)[b]			
Adenine	12.7 (10.0–16.1)	18.0 (14.4–22.5)	1.42 (1.03–1.96)[b]	0.27 (0.12–0.59)	0.89 (0.37–2.14)	3.30 (1.02–10.73)[b]
	12.6 (10.0–15.9)	18.3 (14.9–22.5)	1.45 (1.07–1.97)[b]			
Adenosine	11.4 (8.5–15.3)	17.6 (13.1–25.6)	1.54 (1.02–2.33)[b]	0.18 (0.08–0.39)	0.58 (0.31–1.08)	3.22 (1.18– 8.79)[b]
	14.1 (11.4–17.5)	21.2 (16.2–27.8)	1.50 (1.06–2.13)[b]			
AMP	10.0 (6.7–15.0)	16.9 (12.6–22.7)	1.69 (1.02–2.79)[b]	0.54 (0.34–0.86)	1.22 (0.68–2.20)	2.26 (1.07– 4.79)[b]
ATP	14.1 (11.2–17.8)	24.0 (16.0–36.0)	1.70 (1.06–2.74)[b]	0.17 (0.08–0.34)	0.48 (0.27–0.86)	2.82 (1.11– 7.13)[b]
ADP	15.6 (12.0–20.3)	18.1 (14.6–22.4)	1.16 (0.83–1.62)	0.47 (0.27–0.83)	0.69 (0.42–1.13)	1.47 (0.69– 3.12)
				0.40 (0.24–0.68)	0.50 (0.33–0.77)	1.25 (0.64– 2.45)
2′-deoxyadenosine	14.1 (8.8–22.6)	12.7 (9.0–17.9)	1.11 (0.62–1.98)	0.41 (0.23–0.71)	0.51 (0.32–0.81)	1.24 (0.60– 2.55)
8-bromoadenosine	16.2 (12.3–21.4)	12.9 (10.5–15.9)	1.26 (0.90–1.76)	0.30 (0.15–0.60)	0.41 (0.25–0.66)	1.37 (0.59– 3.19)
	14.3 (9.0–22.7)	16.2 (12.8–20.6)	1.13 (0.67–1.90)			
Hypoxanthine	16.3 (11.9–22.3)	15.2 (11.4–20.2)	1.07 (0.69–1.65)	0.32 (0.21–0.50)	0.35 (0.22–0.55)	1.09 (0.59– 2.03)
	16.1 (12.4–20.9)	21.4 (13.5–34.0)	1.33 (0.78–2.27)			
Uridine	15.2 (11.8–19.6)	13.4 (9.2–19.6)	1.13 (0.72–1.77)	0.51 (0.28–0.94)	0.51 (0.31–0.84)	1.00 (0.45– 2.20)
Cytidine	17.7 (14.2–22.1)	16.5 (13.9–19.6)	1.07 (0.81–1.41)	0.58 (0.36–0.93)	0.26 (0.16–0.42)	2.23 (1.14– 4.35)[b]
				0.41 (0.26–0.65)	0.24 (0.14–0.42)	1.71 (0.83– 3.54)[b]

[a] 50 mg/kg injected ip 18–19 hr prior to morphine assay. Values in parentheses are 95% confidence limits.

[b] Significant at the 5% level.

which use other endpoints in response to pain, and the hot-plate test and the stretching test were chosen. The stretching response is considerably more sensitive to the effects of morphine than either the tail-flick or the hot-plate test.

Ho *et al.* (2) found that consistent antagonism of the antinociceptive action of morphine was observed by the tail-flick test 6–24 hr after 10 mg/kg cAMP was administered iv. In our preliminary experiments, 10 mg/kg cAMP injected either iv or ip 6 hr before the assay occasionally antagonized the antinociceptive action of morphine as tested by either the hot-plate or the stretching test but this result was inconsistent. However, when the dose administered ip was increased to 50 mg/kg and was given at least 16 hr prior to testing, cAMP consistently antagonized the actions of morphine when tested by either procedure. All of the animals used in the experiments reported in Table I, therefore, were injected ip with either 50 mg/kg of the test chemical (weighed in the form in which the drug was obtained) or 0.9% sodium chloride 18–19 hr prior to the beginning of the assay. It is shown in Table I, in which each line gives the data for a separate test, that under these conditions cAMP approximately doubled the ED_{50} of morphine in both tests. This confirms the observation of Ho *et al.* (2) that cAMP administered iv in a lower dose either 6 or 24 hr earlier doubled the AD_{50}[3] of morphine determined by the tail-flick test.

To test the hypothesis that the primary substance responsible for the antagonism of morphine analgesia is a catabolic product of cAMP to which membranes are more permeable, the effects of pretreatment with adenine or adenosine were studied. The results are given in Table I and show that in antagonizing morphine analgesia, both substances were about as effective as cAMP by the hot-plate test and somewhat more effective than cAMP by the stretching test. Other adenine nucleotides which might be expected to form catabolic products similar to those of cAMP were also tested. AMP and ATP were approximately as effective as cAMP in antagonizing morphine analgesia in both types of test. On the other hand, ADP, which should form the same catabolic products as AMP and ATP, did not significantly alter the ED_{50} of morphine in either test. We have no explanation for this discrepancy but it may be significant that ADP appears to have unique effects on membranes as suggested by the fact that ADP induces aggregation of platelets whereas adenosine, AMP, and ATP inhibit ADP-induced platelet aggregation (9).

Two adenosine derivatives, 2′-deoxyadenosine and 8-bromoadenosine, when administered to mice 18–19 hr earlier, did not significantly alter the ED_{50} of morphine, indicating that these particular substitutions in either the ribose moiety or the purine ring of adenosine neutralized its analgesia antagonistic property. The requirement of an intact adenine structure is further suggested by the finding that hypoxanthine did not significantly increase the ED_{50} of morphine. Attempts to test the effects of pretreatment with guanosine were unsuccessful owing to its poor solubility in aqueous media.

Another indication that the purine ring has an important role in the antagonism of morphine analgesia derives from the finding that uridine pretreatment did not reduce the ED_{50} of morphine in either test. Another pyrimidine nucleoside, cytidine, also did not antagonize the action of morphine in either test. Unexpectedly, in the stretching test but not in the hot-plate test, the ED_{50} of morphine for the cytidine-pretreated animals was significantly lower than the ED_{50} for the corresponding controls. We have no explanation for this observation but it was confirmed in a second stretching test assay and appears to be real.

It is clear from the experiments reported here that the antagonism by cAMP of the antinociceptive action of morphine previously reported (1, 2) is not limited to cAMP since it occurred with several other compounds that can be converted to adenine in the body. These results, however, do not exclude the possibility that exogenous adenine,

[3] The AD_{50} (median analgetic dose) is defined as the dose of morphine which delays the tail-flick response to a thermal stimulus by 3.5 sec in 50% of the animals tested 30 min after morphine administration (2).

adenosine, and the adenine nucleotides lead to an increase in the synthesis of cAMP in the central nervous system. The negative results with ADP suggest that some other action of this adenine nucleotide prevented it from producing an adenine-like antagonism of morphine analgesia.

Summary. Antagonism of the antinociceptive action of morphine in mice by prior parenteral administration of cAMP has been confirmed using two additional analgesic testing procedures, the hot-plate test and the stretching test. The analgesia-antagonistic property, however, is not specific for cAMP because it also occurred after pretreatment with compounds which probably increased the concentration of adenosine or adenine in the body. Preliminary indications are that an unsubstituted purine ring is a requirement for the adenine derivative that is primarily responsible for the antagonism of the analgesic action of morphine.

1. Ho, I. K., Loh, H. H., and Way, E. L., Nature (London) **238**, 397 (1972).

2. Ho, I. K., Loh, H. H., and Way, E. L., J. Pharmacol. Exp. Ther. **185**, 336 (1973).

3. Ho, I. K., Loh, H. H., and Way, E. L., J. Pharmacol. Exp. Ther. **185**, 347 (1973).

4. Davoren, P. R., and Sutherland, E. W., J. Biol. Chem. **238**, 3009 (1963).

5. Robison, G. A., Butcher, R. W., Øye, I., Morgan, H. E., and Sutherland, E. W., Mol. Pharmacol. **1**, 168 (1965).

6. Eddy, N. B., and Leimbach, D., J. Pharmacol. Exp. Ther. **107**, 385 (1953).

7. Smits, S. E., and Takemori, A. E., Brit. J. Pharmacol. **39**, 627 (1970).

8. Litchfield, J. T., Jr., and Wilcoxon, F., J. Pharmacol. Exp. Ther. **96**, 99 (1949).

9. Mustard, J. F., and Packham, M. A., Pharmacol. Rev. **22**, 97 (1970).

Received June 21, 1973. P.S.E.B.M., 1973, Vol. 144.