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The Effect of Morphine on Purine and Acetylcholine Release from Rat Cerebral Cortex: Evidence for a Purinergic Component in Morphine's Action

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PHILLIS, J. W., Z. G. JIANG, B. J. CHELACK AND P. H. WU. *The effect of morphine on purine and acetylcholine release from rat cerebral cortex: Evidence for a purinergic component in morphine's action.* PHARMAC. BIOCHEM. BEHAV. 13(3)421–427, 1980.—Morphine enhances the release of adenosine and its metabolites from the rat cerebral cortex and inhibits the release of acetylcholine. Naloxone antagonizes the effects of morphine on both purine and acetylcholine release. The adenosine antagonists, caffeine and theophylline, reduce morphine's effects on acetylcholine release, and at the same time increase the spontaneous release of acetylcholine. It is suggested that morphine, acting at a naloxone-sensitive site, enhances the level of extracellular adenosine, which in turn inhibits the release of acetylcholine, and that some of morphine's actions are mediated by a purinergic step.

Morphine Adenosine Acetylcholine Caffeine Cerebral cortex Release

THE literature contains a number of findings which suggest that methylxanthines (caffeine and theophylline) antagonize the actions of morphine and the endogenous opioids. Theophylline reduces the analgesic effects of morphine in mice [4] and in morphine-dependent rats it enhances the ability of naloxone to precipitate symptoms of morphine abstinence [1]. Both caffeine and theophylline enhance the effects of nociceptive stimulation in rats [13], possibly by antagonizing the actions of endogenous opioids. The theophylline derivative, aminophylline, reversibly reduces the depressant actions of morphine on rat striatal neurons [22]. Methylxanthines antagonize the depressant actions of morphine on acetylcholine (ACH) release from the field stimulated guinea-pig ileum and intact cerebral cortex [6, 7, 20].

Adenosine causes a comparable inhibition of the release of ACH from the field stimulated guinea pig ileal preparation and cerebral cortex [6, 7, 20]. Methylxanthines antagonize this effect of adenosine and at the same time can enhance the rate of efflux of ACH from the intact cortex and isolated cortical slices [7,24]. Methylxanthines also antagonize the depressant effects of adenosine on the firing of cerebral cortical neurons [14,15] and block adenosine stimulation of cyclic 3′,5′-adenosine monophosphate formation in brain preparations [10,19]. The methylxanthines have been extensively used in studies on peripheral tissues, where they appear to act as competitive antagonists of adenosine [12]. Confirmatory evidence that the methylxanthines compete with adenosine for a membrane binding site has recently been obtained in ligand binding studies [11,26].

If, as is indicated by these observations, the methylxanthines exert their effects by blocking a purinergic receptor, the possibility must be considered that morphine reduces ACH release through a purinergic step by enhancing extracellular levels of adenosine. The alternative explanation, namely that morphine and adenosine have a common receptor, is rendered untenable by the observation that the opiate receptor antagonist, naloxone, can prevent the effects of morphine but not those of adenosine [20]. The present experiments were undertaken to see if morphine does increase the rate of efflux of adenosine from the rat cerebral cortex and whether this is associated with a decreased ACH release. The effects of methylxanthines on this morphine-evoked decrease in ACH release were also ascertained. Our finding of a morphine-elicited increase in the rate of efflux of adenosine and its metabolites from the rat cerebral cortex confirms and extends an earlier observation that morphine can increase the veratridine-induced release of purines from rat brain cerebral cortical slices [2]

METHOD

Experiments were carried out on 39 male Sprague-Dawley or Wistar rats (300–700 g wt.). Anaesthesia was induced with halothane and the trachea was cannulated. The animals were then placed in a stereotaxic head holder and anaesthesia was maintained with a mixture of nitrous oxide (75%), oxygen (25%) and methoxyflurane. After completion of the surgical procedures, the methoxyflurane vaporizer was adjusted to ensure that the animals would be lightly but

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adequately anaesthetized and no further changes were made to the flow regulators. Body temperature was kept constant at 37°C by a feedback circuit with a rectal probe. An intravenous cannula was placed in the right femoral vein.

Adenosine Release

Both cortical hemispheres were exposed, leaving a thin crest of bone along the midline. The dura was removed and rectangular cups with inside dimensions of 5×8 mm were placed bilaterally on the cortical surface. Leakage from the cups was prevented by a thin coating of silicone grease on the cup surface in contact with the brain. When the cups were in place, the exposed cortical, bone and muscle surfaces around them were covered with a layer of 4% agar in physiological saline. The cups were filled with a sterile, pyrogen free, physiological saline (Ringer's Injection, Abbott). The solution in each cup was removed and replaced for 40 min with 100 μl of (2,8-^{3}H)-adenosine (0.1 mM; specific activity of 0.1 Ci/M, ICN). In 4 experiments, the cup over one cortex was filled with labelled adenosine and that over the other cortex with (^{14}C)-urea (1.6 mM; specific activity 10 Ci/M, New England Nuclear) for a 40 min incubation period.

At the end of the incubation period, the cups were rinsed ten times in rapid succession with warmed physiological saline and subsequently refilled with 100 μl saline. Thereafter the cup contents were withdrawn every 15 min and replaced with fresh saline. The collected samples were mixed with 2 ml of PCS scintillation fluid and counted in a Nuclear Chicago Isocap 300 liquid scintillation counter.

Ten rats were injected intravenously with 1 mg/kg morphine sulphate (B.D.H.) with a subsequent injection of 5 mg/kg 2 hours later. Five rats were initially administered 0.1 mg/kg naloxone hydrochloride (Endo Drugs Ltd.) followed after 30 min by morphine sulphate (1 mg/kg). Since the efflux of labelled material varied amongst animals, the tabulated results have been standardized by expressing post-drug release values as a percentage of the mean of efflux rates in the two collection periods immediately preceeding drug administration. The effects of the 5 mg/kg injection of morphine sulphate were assessed as a percent change using the release rates in the 30 min period preceeding its administration as the control release. The post drug efflux rates are also presented by pooling them into 30 min time periods. The significance of drug effects on efflux was evaluated using Student's t-test.

Adenosine Metabolism

Four rats were used for an analysis of the metabolism of the ^{3}H-adenosine released into the cortical cups. The cortices of these rats were exposed by removing the bone overlying both hemispheres, including the midline, and a single lucite cylinder (cup) with an inside diameter of 1.1 cm was placed on the dorsal surface of the brain, covering both hemispheres. The cortices of these rats were incubated with 0.5 ml of a solution containing ^{3}H-adenosine (0.1 mM; specific activity of 0.7 Ci/M) for 45 min. After rinsing ten times the cup was refilled with 0.5 ml of physiological saline and collections were made every 15 min as described above. Morphine sulphate (1 mg/kg) was administered intravenously to these rats 2.5 hours after the start of the collection periods. 100 μl of perfusate from each sample was used for counting and the remaining 400 μl was lyophilized and after redissolving in 50 μl distilled water, the material was transferred to the origin of a silica gel thin layer chromatogram (Brinkmann) and developed in the solvent system, n-butanol:ethyl acetate:methanol:NH_4OH (7:4:3:4 V/V), as described by Shimizu *et al.* [21]. The metabolites were located under ultraviolet light. The silica from areas identified as containing adenosine, inosine, adenine, hypoxanthine and nucleotides was transferred to vials and its radioactivity counted.

Acetylcholine Release

Twenty rats were used in these experiments. Bone overlying the cerebral hemispheres was removed, including that in the midline and a single Lucite cup with an inside diameter of 1.1 cm was placed over both hemispheres. Exposed surfaces around the cup were covered with 4% agar in physiological saline and the cup was filled with a solution of 1.65×10^{-4}M neostigmine bromide in physiological saline. This solution was changed three times at 20 min intervals and the cup was then rinsed several times and filled with 0.6 ml of the neostigmine-containing solution. This solution was collected after 15 min and replaced with fresh solution. Subsequent collections were made at 15 min intervals. Morphine sulphate (0.5 or 2.0 mg/kg) was administered intravenously immediately after the sixth collection. The ACH content of each cortical perfusate was determined by bioassay on the hearts of *Mercenaria mercenaria* [5]. At the end of each experiment the hearts were perfused with benzoquinonium chloride (5×10^{-7}M; Mytolon, an ACH antagonist)-containing sea water and in every instance the inhibitory effects of the cortical perfusates were abolished.

Adenosine, adenosine 5′-monophosphate and adenosine 5′-triphosphate were tested on six *Mercenaria* hearts at concentrations of 10^{-6}, 10^{-5} and 10^{-4}M. No effects of these compounds were observed on the rate or force of contraction of these hearts.

RESULTS

Metabolism of Adenosine

The distribution of radioactivity in the products of adenosine uptake and metabolism was determined in the cortical perfusates of four rats after incubation with labelled adenosine. The bulk of the labelled material (50–75%) in all the perfusates was in the form of nucleotides, including cyclic AMP. Inosine accounted for most of the remaining labelled material (15–40%) with adenosine and hypoxanthine together accounting for about 10% of the total activity. A small amount (1–2%) of labelled adenine was also detected in the samples. Morphine sulphate (1 mg/kg) administration did not alter the relative proportions of the various metabolites in the cortical perfusates. Although the proportion of the various metabolites released from the cortex of any one animal tended to remain constant throughout the experiment, there was considerable variability between animals.

Purine Release

The release of adenosine and its metabolites from 26 cortices (15 animals) was studied. The amount of labelled material released showed an exponential decline which tended to reach a plateau phase 2.5 hours after the end of the incubation period. Morphine or naloxone were therefore administered 150 min after the start of sample collecting (i.e. after removal of the tenth sample).

Figure 1 illustrates an experiment in which morphine sul-

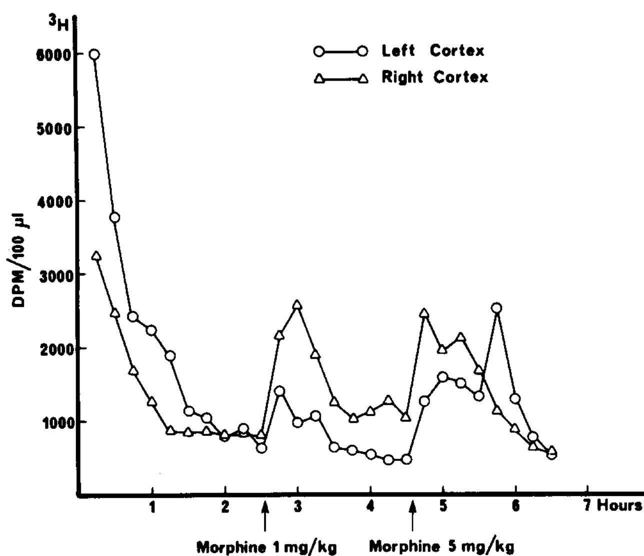

FIG. 1. Efflux of labelled adenosine derivatives from right and left rat cerebral cortices. After preincubation with ^{3}H-adenosine for 40 min, purine efflux studies were carried out for 6.5 hours. The cup contents were removed for counting every 15 min and replaced with 100 μl of physiological saline. After 2.5 hours (at point indicated by the first arrow) morphine sulphate (1 mg/kg) was administered intravenously. A second dose of morphine sulphate (5 mg/kg) was administered 2 hours after the first. Collections were continued for a further 2 hours.

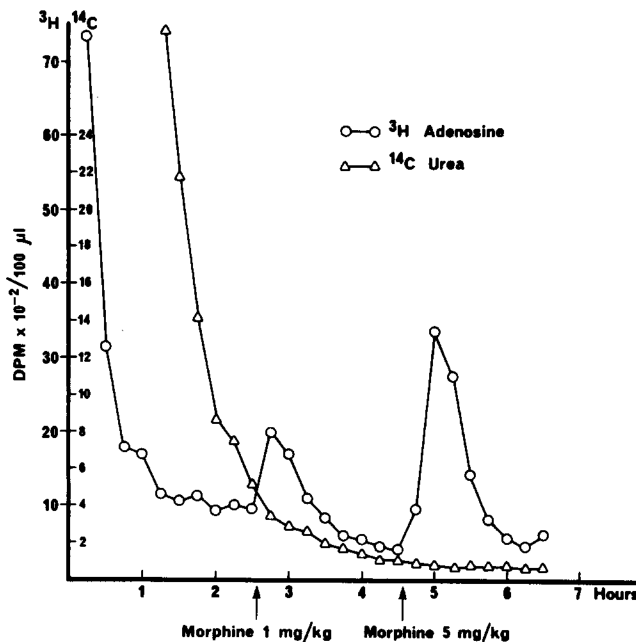

FIG. 2. Efflux of ^{3}H-labelled purines and ^{14}C-labelled urea from the left and right cortical hemispheres of a rat brain. During the loading period the left cup contained ^{3}H-adenosine and the right cortical cup ^{14}C-urea. Collections were made at 15 min intervals as described for Fig. 1. Morphine sulphate (1 and 5 mg/kg) was administered at times indicated by the arrows.

phate, 1 mg/kg and then 5 mg/kg, was administered intravenously. The rate of ^{3}H-efflux declined rapidly from the immediately post-incubation levels and had reached a stable phase 2 hours later. Morphine was injected at 150 min and caused an increase in the rate of efflux of label from both hemispheres, which continued for several collection periods. The second injection of morphine, 2 hours after the first administration, elicited a more pronounced, and long lasting, release of labelled purines. The rate of release then returned to pre-morphine levels.

In four experiments ^{14}C-urea replaced ^{3}H-adenosine in one of the cups and the effects of morphine on adenosine and urea release (used as an indicator of non-specific alterations in release) could be compared. The results of one of these experiments is shown in Fig. 2. The rate of urea efflux declined in parallel with that of the purines (as dpm), but the release of urea unlike that of the purines was not affected by morphine (1 and 5 mg/kg). The rate of urea release was unaffected by morphine in the other three experiments.

The results obtained following morphine administration

TABLE 1

EFFECTS OF MORPHINE AND NALOXONE ON ^{3}H-PURINE EFFLUX FROM THE RAT CEREBRAL CORTEX

A	Morphine (1 mg/kg)			Morphine (5 mg/kg)			
Control[1]	0–30 min	30–60 min	60–90 min	Control	0–30 min	30–60 min	60–90 min
100(16)[2]	144 ± *15.8	127 ± 13.5	93 ± 7.4	100	189 ± †26.3	164 ± *26.8	116 ± 22.0

B	Naloxone (0.1 mg/kg)	Morphine (1 mg/kg)	
Control	0–30 min	0–30 min	30–60 min
100(10)	114 ± 13.7	95 ± 8.7	100 ± 13.6

[1]Control release (100%) is the average ^{3}H-efflux rates (in dpm) during the two 15 min periods immediately preceeding morphine administration. Post morphine efflux rates are derived from the averages of pairs of successive 15 min collection periods during three consecutive 30 min periods.

[2]Figures in parentheses represent the number of cortices from which efflux was measured.

[3]Values are expressed as $\bar{x}$ ± SEM.

*$0.01<p<0.05$.

†$0.001<p<0.01$.

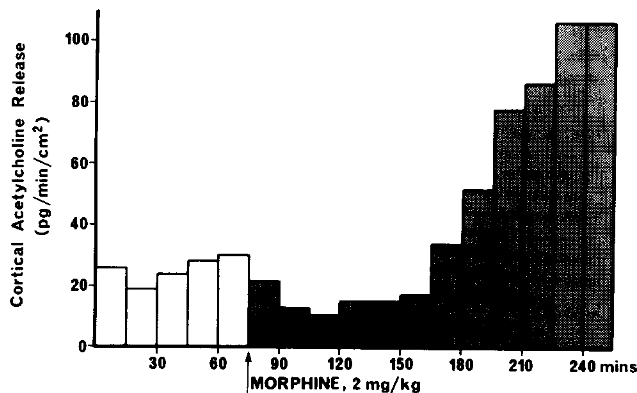

FIG. 3. Rates of ACH release from a rat cerebral cortex (the cup covered portions of both hemispheres) before and after intravenous administration of morphine sulphate (2 mg/kg). Each collection period was of 15 min duration.

to ten rats (16 cortices) are summarized in Table 1A. At a dose level of 1 mg/kg, morphine sulphate evoked an increase in purine efflux from 12 of the 16 cortices and at 5 mg/kg, there was an increased efflux from 15 of the 16 hemispheres. The increases in purine efflux were more pronounced and long-lasting when the larger dose of morphine was administered.

Naloxone hydrochloride (0.1 mg/kg) was administered intravenously to five rats followed after 30 min by morphine sulphate (1 mg/kg). Naloxone evoked a small, but not significant increase in labelled purine efflux from the cerebral cortex. Morphine sulphate (1 mg/kg), given 30 min after naloxone, had no effect on the efflux of labelled material.

Acetylcholine Release

Cortical ACH release into a cup overlying both cerebral cortices was relatively uniform in any given animal, although tending to increase slowly during the several hours that it took to complete each experiment. The control rate of release varied from animal to animal with a mean for all animals of 50.27±17.25 pg/min/cm^2 (mean±SEM).

Morphine sulphate (0.5 and 2 mg/kg) depressed cortical ACH release. Figure 3 shows the decrease in ACH release from control levels following an injection of morphine sulphate (2 mg/kg) to one rat. The onset of depression of release was immediate and reached its maximum 30–45 min after morphine was administered. Release returned to control levels after 1.5 hours and was then apparently enhanced. The averaged results of 5 experiments are presented in Fig. 4. Morphine reduced ACH release in the three 30 min time periods following its administration, with a maximum reduction to 60 percent of control. The rate of ACH release then recovered to control levels. The different rates of recovery, and tendency for release to exceed control levels in some experiments, is the cause of the large standard errors of the means in the last two 30 min time periods. The delayed enhancement of ACH release observed in some experiments (Fig. 3), may be a result of the same action of morphine which is responsible for the increase in ACH release seen when morphine is administered in unanaesthetized animals [16].

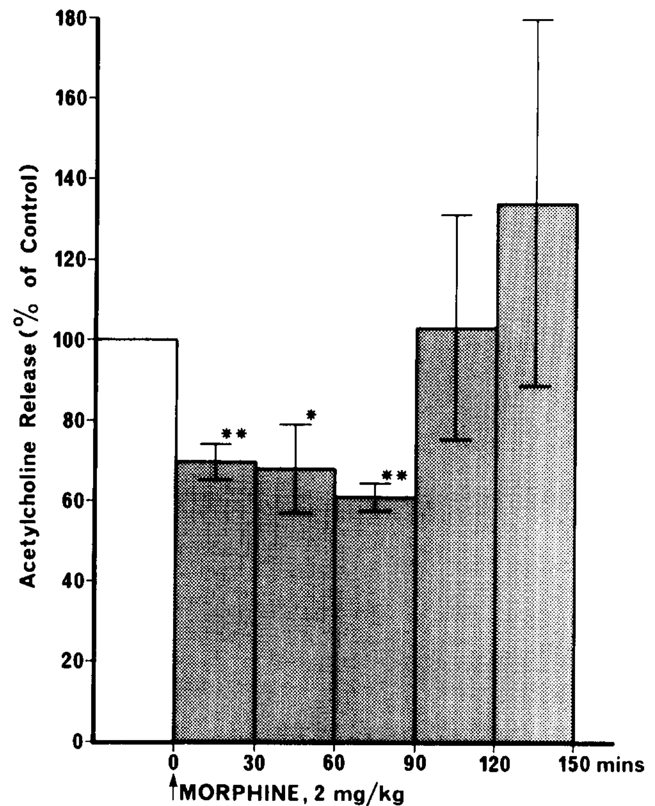

FIG. 4. Cortical ACH release following intravenous administration of morphine sulphate (2 mg/kg). Each histogram is the average of ACH release data obtained from the 6 animals used in these experiments and represents a 30 min time period (two 15 min samples). ACH release is presented as a percent of the control value, which is set at 100% and corresponds to 94.78±54.78 (pg/min/cm^2; mean±SEM). The rate of ACH release was depressed by morphine with respect to the reference rate at *$p<0.05$, **$p<0.01$. (Student "*t*" test).

Morphine sulphate (0.5 mg/kg) also reduced ACH release. The results of 5 experiments are illustrated in Fig. 5. Morphine caused a reduction in release during the 60 min period following its administration. At this point, the animals were administered theophylline (20 mg/kg) intravenously which evoked a rapid increase in ACH release to more than twice the control rate.

In another series of experiments, rats were given caffeine citrate (20 mg/kg, 4 rats; 40 mg/kg, 6 rats) intravenously followed after 45 min by morphine sulphate (2 mg/kg). The results from an individual experiment are presented in Fig. 6. Caffeine citrate (40 mg/kg) caused an immediate increase in ACH release, which had nearly trebled 30–45 min after caffeine administration. Morphine administered at this point caused an initial small reduction in the rate of ACH release and then 60 min later there was a further marked increase in release.

The results of all these caffeine experiments are summarized in Fig. 7. Caffeine citrate (20 mg/kg) evoked a large increase in ACH release. Morphine reduced the amount of the increase during the 60 min period after its administration (but the reduction was not significant) and then release increased again. Caffeine citrate (40 mg/kg) administration re-

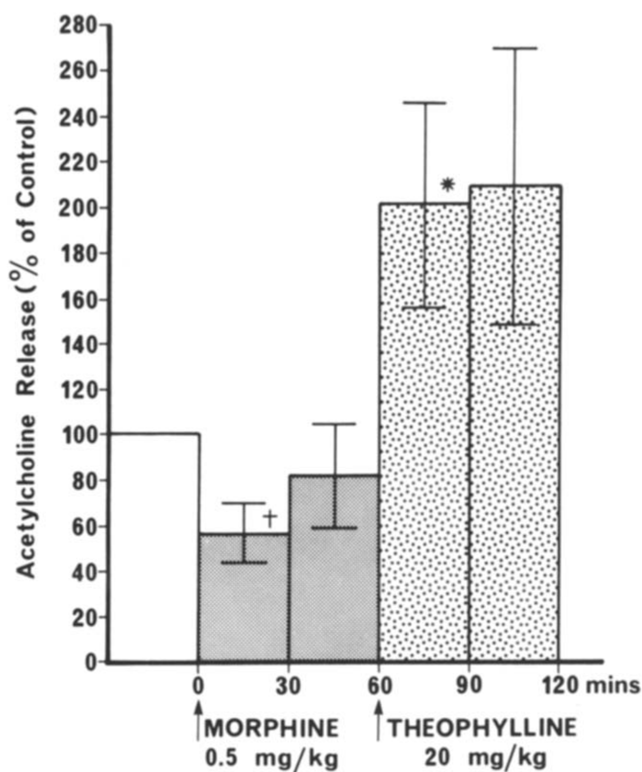

FIG. 5. Cortical ACH release following intravenous administration of morphine sulphate (0.5 mg/kg) and theophylline (20 mg/kg). The histograms are averages of ACH release from 4 animals, as described in Fig. 4. The reference rate of release was 28.66±8.71 (pg/min/cm²; mean±SEM). Morphine decreased, and theophylline increased ACH release with respect to the reference rate of †$p=0.05$, *$p<0.05$.

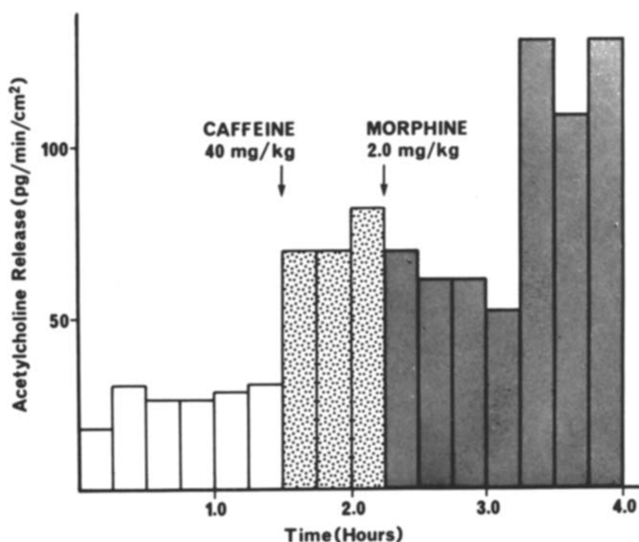

FIG. 6. ACH release from an individual rat cerebral cortex. Caffeine citrate (40 mg/kg), intravenously administered, increased ACH release. Morphine sulphate (2.0 mg/kg), administered 45 min after caffeine, caused an initial small reduction (lasting 60 min) after which there was a further increase in release.

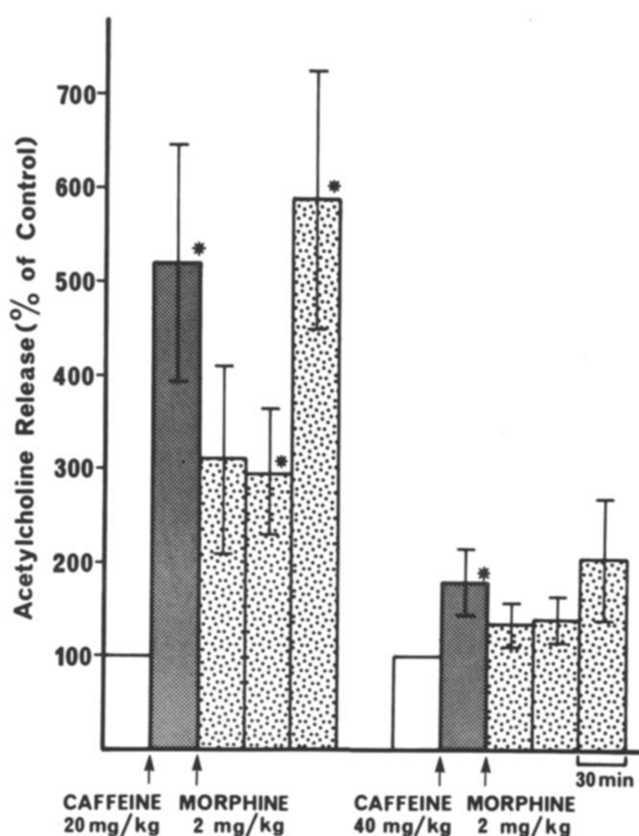

FIG. 7. Effect of intravenously administered caffeine citrate (20 and 40 mg/kg) and morphine sulphate (2 mg/kg) on ACH release from the rat cerebral cortex. Caffeine citrate (20 mg/kg) was administered to 4 rats; 40 mg/kg to 6 rats. ACH release is represented as a percentage of the control value which is set at 100%, and corresponds to 29.97±8.32 pg/min/cm²; mean±SEM) for the first group and 37.02±11.43 pg/min/cm²; mean±SEM for the second group. *indicates that release was significantly above the reference rate ($p<0.05$). The reduction in caffeine-enhanced release rates after morphine administration are not statistically significant when compared by a one way analysis of variance.

sulted in a small, but significant, increase in ACH release and in these animals, morphine sulphate (2 mg/kg) caused a small, not significant, reduction which lasted for 60 min.

DISCUSSION

The experiments described in this report show that morphine affects the release of adenosine and its derivatives and ACH from the *in vivo* rat cortex. Purine release was measured by prelabelling the endogenous pools with ^{3}H-adenosine and endogenous ACH release was measured by a sensitive and reliable bio-assay technique (the *Mercenaria mercenaria* heart). Following such prelabelling with labelled adenosine most of the radioactive marker is associated with adenine nucleotides and adenosine [8]. After release the label is associated with nucleotides, adenosine and its metabolites (inosine and hypoxanthine) ([8, 9, 23] present results).

Morphine increased the rate of efflux of purines from the *in situ* rat cerebral cortex whilst urea release, which was

used as an indicator of non-specific alterations in release, was unaffected. The alterations in purine release therefore reflect a selective action of morphine. The effect is unlikely to have been a consequence of an increase in the depth of anaesthesia and/or subsequent fall in oxygen tension as no increases in adenosine release were observed in other experiments after the administration of pentobarbital sodium in doses of up to 15 mg/kg [17]. The observation of a morphine-elicited increase in purine efflux from isolated perfused rat cerebral cortical slices [2] is consistent with this conclusion.

The purine-release enhancing effect of morphine could be due either to an increase in purine release or inhibition of adenosine and inosine uptake. Morphine (150 μM) inhibits the uptake of adenosine (1 μM) into rat brain synaptomsomes by more than 50 percent (unpublished observations) and this action may account for its effect on purine efflux from the intact brain.

A naloxone-antagonized inhibitory effect of morphine on ACH release from the cerebral cortex of anaesthetized rats has been reported previously [6,7]. Caffeine and theophylline also antagonized the effects of morphine. In the present experiments, theophylline (20 mg/kg) antagonized the reduction in release elicited by morphine and after caffeine (20 and 40 mg/kg) morphine no longer elicited a significant reduction in ACH release. Caffeine and theophylline increase the rate of ACH efflux from the rat cerebral cortex [17], an effect which is likely to be a result of the antagonism between the methylxanthines and endogenously released adenosine. An interesting observation is that the lower (20 mg/kg) dose of caffeine evoked a greater increase in ACH release than did the 40 mg/kg dose. Depression of central nervous system function by large doses of caffeine has been described by Waldeck [25], who reported that caffeine 25 mg/kg administered intraperitoneally increased locomotor activity in mice by almost 100 percent above the normal level. No further increase in activity was observed after 50 mg/kg, and after 100 mg/kg locomotor activity was reduced to below control levels. The depressant action of large amounts of caffeine may account for the smaller increase in ACH release observed in the present experiments.

In conclusion, the results in this report confirm earlier findings that morphine can both enhance adenosine efflux from rat cerebral cortical tissues and depress ACH release. Morphine's effects on ACH release are antagonized by the adenosine antagonists, caffeine and theophylline. The most plausible explanation for these results is that morphine enhances extracellular adenosine levels, possibly by preventing its uptake, and that the depression of ACH release is a consequence of the enhanced extracellular adenosine levels. It is difficult on the basis of the existing evidence to exclude other explanations, such as the possibility that adenosine and morphine reduce transmitter output by immobilizing Ca^{++} and that the methylxanthines oppose their actions by releasing intracellular Ca^{++}. Our finding of an increase in adenosine release, which parallels the decrease in ACH release, appears however to be consistent with the opinion that at least part of morphine's effect is mediated by adenosine. Circumstantial support for this suggestion is provided by the report that the inhibitory effects of morphine on the contractions of the field stimulated guinea-pig ileum are potentiated by dipyridamole, a potent adenosine uptake inhibitor [3]. Adenosine mediation of some of morphine's action would also provide an explanation for morphine's stimulant action on adenylate cyclase [18] since adenosine is well known to be a potent stimulant of cyclic 3′,5′-adenosine monophosphate formation.

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