Br. J. Pharmac. (1983), **80**, 727–734

Antagonism of enkephalin action on acetylcholine release by methylxanthines: lack of a purine link

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1 Theophylline (Theo) and caffeine antagonized the inhibitory effect of methionine (Met)-enkephalin, leucine (Leu)-enkephalin and morphine on the twitch height of the field stimulated myenteric plexus longitudinal muscle (MPLM) preparation of the guinea-pig ileum. Antagonism by Theo was observed only in tissues stimulated submaximally, but that by caffeine was observed in tissues stimulated submaximally and supramaximally.

2 Injection of Theo (20, 40 mg kg^{-1}) or caffeine (40 mg kg^{-1}) reversed or blocked the inhibitory effects of Leu-enkephalin (50 μg i.c.v.) and a systemically active enkephalin FK 33,824 (0.5 mg kg^{-1}) on the release of acetylcholine (ACh) from the rat cortex *in vivo*.

3 Injections of morphine (2.5, 5.0 mg kg^{-1} i.v.) did not modify the *in vivo* release of radiolabelled purines from the cerebral cortex prelabelled with [^{3}H]-adenosine (2.8×10^{-7} M). Application of K^+ (60 mM) to the cortex readily stimulated this release. Injection of morphine (5.0 mg kg^{-1} i.v.) increased the spontaneous release of radiolabelled purines from the cortex prelabelled with a higher concentration of [^{3}H]-adenosine (10^{-4} M) in six out of eleven experiments. Under similar conditions neither Leu-enkephalin (50 μg i.c.v.) nor FK 33,824 (0.5 mg kg^{-1} i.v.) stimulated purine release.

4 It is concluded that methylxanthines can antagonize the inhibitory action of opioids on the peripheral and central release of ACh. However, this antagonism does not reflect an intermediary purine step in the action of opioids on the release of ACh.

Introduction

Morphine-related opiates and enkephalins act on specific opiate receptors to depress the peripheral and central release of acetylcholine (ACh) (Jhamandas & Sutak, 1974; 1976; 1980; Jhamandas, Hron & Sutak, 1975). The inhibitory actions of morphine on the release of ACh from the guinea-pig ileum or the rat cerebral cortex can be selectively antagonized by the methylxanthines, theophylline (Theo) and caffeine (Jhamandas & Sawynok, 1976; Sawynok & Jhamandas, 1976; 1979). Since methylxanthines block a certain class of adenosine receptors (Burnstock, 1978; Daly, Burns & Snyder, 1981), the antagonism of morphine's action by these agents suggested that adenosine, released by morphine, may mediate the inhibitory actions of this opioid agonist on the release of ACh. This suggestion is apparently supported by the findings that adenosine inhibits the peripheral and central release of ACh and its action is blocked by methylxanthines but not by naloxone (Jhamandas & Sawynok, 1976; Sawynok & Jhamandas, 1976; Vizi & Knoll, 1976). Furthermore, morphine has been reported to increase the release of tritium-labelled adenosine and its derivatives (hereafter referred to as radiolabelled purines) from the cerebral cortex *in vitro* and *in vivo* (Phillis, Jiang, Chelak & Wu, 1980).

However, a number of studies have questioned the involvement of adenosine in the inhibitory actions of morphine on ACh release. In some investigations Theo failed to antagonize the inhibitory action of morphine on the response of the guinea-pig myenteric plexus-longitudinal muscle (MPLM) preparation (Gallant & Clement, 1981; Vizi, Somogyi & Maygar, 1981), although in other studies this antagonism was clearly observed (Brailowsky, Guerrero-Munoz, Lujan & Shkurovich, 1981). The enzyme adenosine deaminase, which catalyses breakdown of adenosine and abolishes its inhibitory effect on the ileum, failed to block the inhibitory action of morphine in this tissue (Jhamandas & Sawynok, 1976). In other experiments local application of morphine, levorphanol or an analogue of enkephalin failed to increase release of radiolabelled purines from the cerebral cortex *in vivo* (Jhamandas & Dumbrille, 1980). In view of these conflicting reports, the status of adenosine as an intermediary in

the anti-release action of morphine and other opioids on ACh remains uncertain and requires clarification.

The discrepant findings arising from the above methylxanthine studies might be due to different experimental conditions employed by various investigators. In an attempt to resolve the differing opinions, we have re-examined the methylxanthine antagonism of opioids and the effect of opioids on the *in vivo* release of radiolabelled purines using different experimental conditions. Considering that observations made on morphine might also apply to the naturally occurring opioid peptides, experiments performed in this study have included both morphine and enkephalins as the opioid agonists. The inclusion of enkephalins was also motivated by the possibility that methylxanthines may exert some of their pharmacological actions by interfering with the endogenous opioid systems. The specific objectives of this investigations were to determine (a) if Theo and caffeine antagonize opioid action on the electrically stimulated guinea-pig MPLM preparation and whether this antagonism is influenced by the intensity of stimulation; (b) if Theo and caffeine antagonize the inhibitory action of enkephalins on the *in vivo* release of cortical ACh; (c) if morphine and enkephalins induce release of radiolabelled purines from the rat cerebral cortex *in vivo*.

Methods

The methods used to test the methylxanthine antagonism of opioid action on the peripheral and central release of ACh and the action of opioids on the *in vivo* release of purines from the rat cerebral cortex *in vivo* have been described in considerable detail in previous papers from this laboratory (Jhamandas & Sutak, 1974; Sawynok & Jhamandas, 1976; Jhamandas & Dumbrille, 1980). Therefore, only a brief description of these methods is presented here.

Twitch experiments on the guinea-pig myenteric plexus longitudinal muscle preparation

Since in previous work Theo was found to antagonize the action of morphine in MPLM preparations stimulated submaximally (Sawynok & Jhamandas, 1976) but not in those stimulated supramaximally (Gallant & Clement, 1981; Vizi *et al.*, 1981), the methylxanthine antagonism experiments in this study were performed using both conditions of electrical field stimulation. The tissue preparation, stimulation and recording of the twitch was carried out by the method described by Sawynok & Jhamandas (1976). In tests involving submaximal field stimulation the level of stimulation required to produce a maximal twitch height was first determined. The stimulus voltage was subsequently adjusted downward to produce a response 60 or 80% of the maximum twitch height. The intensity of the stimulus required to produce either level of submaximal response was determined for each tissue preparation at the start of an experiment and held constant for the duration of the experiment. Supramaximal stimulation was applied at 80 V. In each tissue preparation only one level of stimulation was used. Methylxanthines, Theo or caffeine, were added to the tissue bath (5 ml volume) 2 min before the addition of an opioid agonist. Dose-response curves, in the absence or presence of the methylxanthines, were constructed as described in our earlier study.

Cortical acetylcholine release

The spontaneous release of ACh from the rat cortex was measured using the cortical cup technique (Jhamandas & Sutak, 1974). ACh released in the presence of the anticholinesterase inhibitor, neostigmine, was assayed biologically on the clam heart preparation. In drug experiments, all agents except Leu-enkephalin were administered into the femoral vein. Leu-enkephalin injections (5 μl volume) were made into the left or right lateral ventricle through a 30 gauge cannula. The patency of this cannula and the delivery of solution into the ventricular space was confirmed by a dye injection at the end of each experiment.

Release of radiolabelled purines from the cortex in vivo

The effect of opioids on the *in vivo* release of radiolabelled purines from the rat cerebral cortex prelabelled with [^{3}H]-adenosine was tested using the cup method described by Jhamandas & Dumbrille (1980). The cerebral cortex was prelabelled with a low (2.8×10^{-7} M) or a high (10^{-4} M) concentration of [^{3}H]-adenosine since in previous work different effects of morphine have been reported under these two conditions. Details of surgery, prelabelling the cortex, collection of samples and identity of the released label have been described fully in the earlier study. The opioid agents tested in the present study were administered intravenously or intracerebroventricularly (i.c.v.). To ensure that failure to observe a drug effect on release was not due to an unreliable cortical tissue preparation, a high concentration of K^+ (60 mM) was applied to the cerebral cortex during the last collection period. The ability of K^+ to induce a significant release of radioactivity over the baseline was taken as evidence for a viable preparation. Experiments in which the cortex showed the slightest trauma, or appeared visibly pale, were discarded. Only a single drug and dose was tested in each animal.

Statistics

Statistical analysis was carried out on the data using an unpaired Student's *t* test. A value of P less than 0.05 was considered to be significant.

Drugs and solutions

Materials used were: [^{3}H]-adenosine (sp. act. 30–35 Ci mmol^{-1}, New England Nuclear Corporation, U.S.A.), FK 33,824 (Sandoz, A.G., Basel, Switzerland), leucine (Leu) enkephalin (Peninsula Laboratories, California, U.S.A.) and morphine sulphate (BDH Pharmaceuticals).

All drugs to be tested were dissolved in saline for intravenous or i.c.v. injection. Solutions of high concentration of K^+ (60 mM) were prepared by replacing sodium in the Ringer-Locke solution with an equivalent amount of K^+.

Results

Twitch experiments

The effects of opioids on the electrically evoked twitch of the guinea-pig MPLM preparation in the absence and presence of a methylxanthine agent are shown in Figures 1 to 3. Figure 1 illustrates the action of Theo or caffeine on the Met-enkephalin-induced inhibition of the twitch height in tissues stimulated submaximally (to produce 60 and 80% maximum response) or supramaximally. The tissues stimulated submaximally showed a greater sensitivity to the inhibitory effect of Met-enkephalin than those stimulated supramaximally, as is indicated by a lower threshold dose and a higher magnitude of the maximal response (Figure 1a, b). The Met-enkephalin dose-response curve obtained in tissues stimulated submaximally was shifted to the right in the presence of Theo (10^{-4} M) indicating an antagonism of the opioid agonist by this drug (Figure 1a, b). Theo failed to produce a significant rightward shift in the Met-enkephalin dose-response curve when the tissues were stimulated supramaximally (Figure 1c). In similar experiments when caffeine (10^{-4} M) was employed as the antagonist, the enkephalin dose-response curve was shifted to the right regardless of the intensity of stimulation applied to the tissues (Figure 1d–f). A visual inspection of the dose-response curves shows that the antagonism of Met-enkephalin by the methylxanthines was greatest in

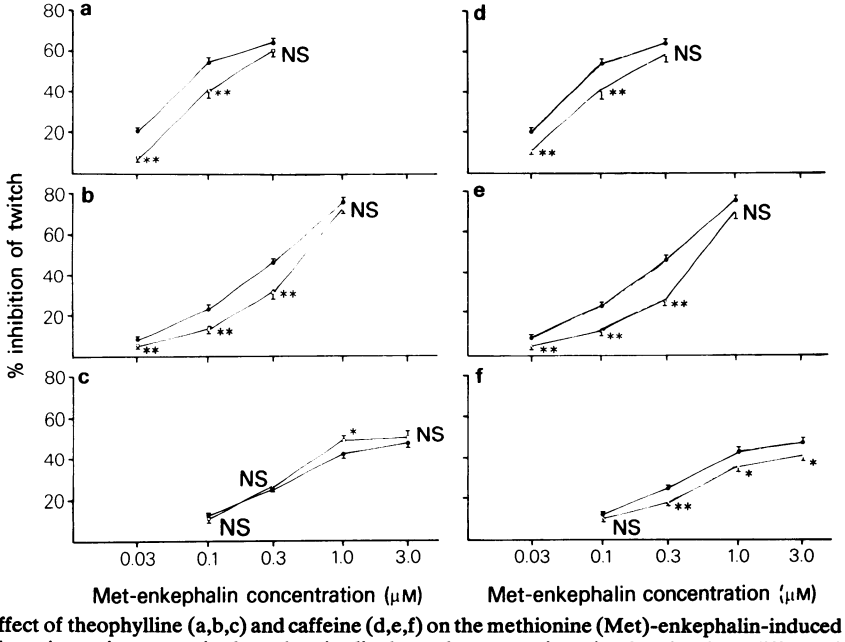

Figure 1 Effect of theophylline (a,b,c) and caffeine (d,e,f) on the methionine (Met)-enkephalin-induced inhibition of twitch in the guinea-pig myenteric plexus longitudinal muscle preparation stimulated at three different intensities. Tissues were stimulated electrically at voltages producing the following twitch heights: 60% maximum (a and d), 80% maximum (b and e) and maximum (c and f; supramaximal stimulation: 80 V). In each section the dose-response curves shown on the right (controls) are in the absence of a methylxanthine and those on the left are in its presence. Theophylline or caffeine (10^{-4} M) was added 2 min before the addition of Met-enkephalin. Values shown are mean ± s.e. from four to twelve separate experiments. Significance of difference from the control is indicated by $^{**}P = <0.01$; $^{*}P = <0.05$. NS = not significantly different from control.

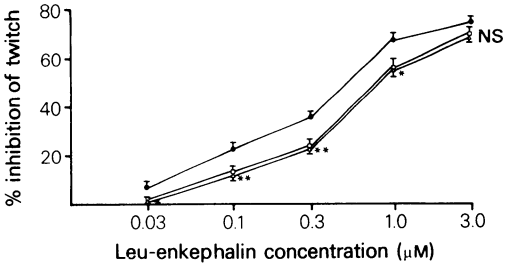

Figure 2 Effect of theophylline (△) and caffeine (□) (10^{-4} M) pretreatment on the inhibition of twitch of the guinea-pig myenteric plexus longitudinal muscle preparation. Tissues were stimulated at voltage producing 80% of the maximal twitch height. Values shown are mean ± s.e. from four separate experiments. Significance of difference from the control (●) is indicated by $^{**}P = < 0.01$, $^{*}P = < 0.05$.

tissues that were stimulated to produce 80% of the maximal response (Figure 1b, e).

The ability of the methylxanthines to antagonize the action of non-opioids on the twitch inhibition was

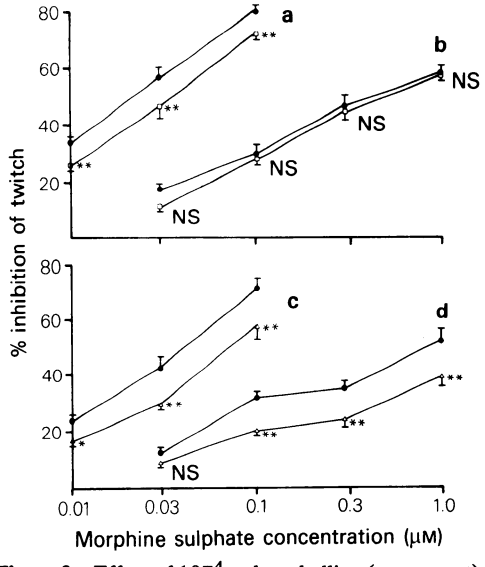

Figure 3 Effect of 10^{-4} M theophylline (upper part) or caffeine (lower part) pretreatment on the morphine-induced inhibition of twitch in the guinea-pig myenteric plexus preparation stimulated submaximally (a and c) and supramaximally (b and d). Dose-response curves in (a) and (c) were derived in tissues stimulated to produce 80% maximal response. Tissues in (b) and (d) were stimulated supramaximally (80 V). In each case (a to d) the control dose-response curve is shown on the left. Values shown are mean ± s.e. of four to six separate experiments. Significance of difference from control is indicated by $^{**}P = < 0.01$. NS =not significantly different from control.

not tested here, but in a previous study it was found that the dose of Theo used does not modify the inhibition produced by dopamine (Sawynok & Jhamandas, 1976). The latter study also showed that at this dose, Theo does not modify contractions of the unstimulated MPLM preparation elicited by exogenous ACh. In electrically stimulated preparations Theo or caffeine by themselves caused a 5 or 10% increase in the twitch height.

Figure 2 illustrates results of experiments with Leu-enkephalin in tissues stimulated to produce 80% of maximal twitch height. As shown, pretreatment with Theo or caffeine produced a small but significant rightward shift in the Leu-enkephalin dose-response curve, indicating an antagonism by the two methylxanthine agents. In similar experiments, shown in Figure 3, Theo produced a rightward shift in the morphine dose-response curve obtained in tissues stimulated submaximally, but not in tissues stimulated supramaximally. Pretreatment with caffeine produced a rightward shift in the morphine dose-response curve under both conditions of stimulation. Thus, in twitch experiments involving morphine and Met-enkephalin, the antagonistic action of Theo against these two opioids was seen only if a submaximal stimulation was used. In contrast, a similar action of caffeine was observed regardless of whether a submaximal or a supramaximal stimulation was employed.

Cortical acetylcholine release

Figures 4 and 5 show cumulative results of experiments in which the ability of methylxanthines to antagonize the action of enkephalins on cortical release of ACh was examined. The two enkephalins used in this part of the study previously have been demonstrated to inhibit cortical ACh release by a naloxone-sensitive mechanism (Jhamandas & Sutak, 1980). As shown in Figure 4, a single injection of Leu-enkephalin depressed the resting output of cortical ACh by about 50%. Administration of caffeine (40 mg kg^{-1}) 20 min after the enkephalin injection restored the release of ACh to 80% of the pre-enkephalin control value (Figure 4a). Pretreatment of the animal with the same dose of caffeine completely blocked the depressant action of Leu-enkephalin on ACh release (Figure 4b). Theo produced effects identical to those observed with caffeine, except that its effects were observed at half the dose (20 mg kg^{-1}) (Figure 4c, d). In these experiments neither Theo nor caffeine by itself influenced the basal release of ACh.

Figure 5 illustrates the effects of methylxanthines against a systemically active enkephalin analogue, FK 33,824, which previously has been found to produce a sustained inhibition of cortical ACh release

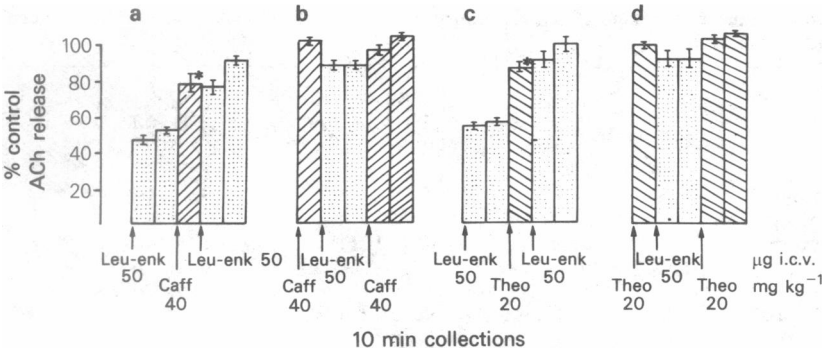

Figure 4 Reversal or blockade of the inhibitory action of leucine enkephalin (Leu-enk) on cortical acetylcholine (ACh) release by caffeine (Caff) or theophylline (Theo). Leu-enk was injected into the lateral ventricle in 5 μl volume. Caff and Theo were administered intravenously into the femoral vein. The values of control release, represented as average release in the two collections preceding the first injection, were: from left to right, (a) 2.2 ± 0.2 ($n = 4$); (b) 2.4 ± 0.1 ($n = 4$); (c) 2.2 ± 0.2 ($n = 4$), 2.4 ± 0.2 ng $10\,min^{-1}$ $0.25\,cm^{-2}$. Each vertical column is mean ± s.e.

following a single dose (Jhamandas & Sutak, 1980). In the present tests a 0.5 mg kg^{-1} dose of FK 33,824 depressed the spontaneous release of ACh by about 40% (Figure 5a). Theo (20 mg kg^{-1}) administered 10 min after the injection of FK 33,824 produced a partial reversal of the enkephalin-induced depression (Figure 5b). A higher dose (40 mg kg^{-1}) produced a more complete reversal of the enkephalin effect, and pretreatment with this dose abolished the inhibitory action of the enkephalin on the cortical ACh release (Figure 5c, d). Injection of caffeine (40 mg kg^{-1}) produced a partial reversal of the enkephalin effect (Figure 5c). Thus, in comparison with Theo, caffeine had a weaker antagonistic action against this opioid agent.

Cortical release of radiolabelled purines

The results of purine release experiments performed under two prelabelling conditions are represented in Table 1. In experiments on the cerebral cortex preloaded with a low [^{3}H]-adenosine concentration (2.8×10^{-7} M), intravenous injection of morphine (2.5 and 5.0 mg kg^{-1}) failed to modify the spontaneous release of radioactivity from this region. In such experiments a topical application of K^+ consistently stimulated the release of radiolabelled purines. When the action of intravenous morphine (5.0 mg kg^{-1}) was tested in experiments in which the cortex had been prelabelled with a higher [^{3}H]-adenosine concentration (10^{-4} M), this drug pro-

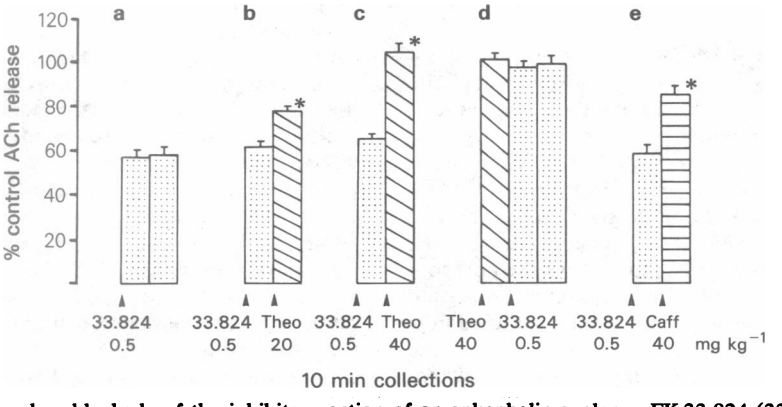

Figure 5 Reversal or blockade of the inhibitory action of an enkephalin analogue FK 33,824 (33,824) on the cortical release of acetylcholine (ACh) by theophylline (Theo) or caffeine (Caff). All drugs were administered intravenously. The values of control release, representing average release in the two collections preceding the first injection, were in the range 2.2 to 2.4 ± 0.2 ng $10\,min^{-1}$ $0.25\,cm^{-2}$. Each vertical column is mean ± s.e. of three to four separate experiments.

Table 1 Effects of morphine, enkephalins and potassium on the spontaneous release or radiolabelled purines from the rat cerebral cortex *in vivo*

Treatment	*Dose*	*% basal release** [^{3}H]-*adenosine* ($2.8 > 10^{-7}$ M)**	[^{3}H]-*adenosine* (10^{-4} M)**
Saline		95 ± 10 (6)	92 ± 10 (3)
Morphine	2.5 mg/kg i.v.	98 ± 10 (4)	—
Morphine	5 mg/kg i.v.	105 ± 10 (18)	213 ± 146 (11)***
Leu-Enkephalin	50 μg i.c.v.	—	102 ± 6 (10)
FK 33,824	0.5 mg/kg i.v.	—	99 ± 9 (8)
Potassium	60 mM (topical)	326 ± 12 (4)****	344 ± 45 (15)****

* Baseline release (100%) represents the average release (d.p.m.) of radioactivity during two 15-min collection periods preceding injection or application of the drug. Values shown are average release during two 15-min collections immediately following drug treatment expressed as a percentage of baseline release. Each value is mean ± s.e. Number of experiments in parentheses.

** The cerebral cortex was prelabelled with 2.8×10^{-7} or 10^{-4} M [^{3}H]-adenosine.

*** Significantly different from saline treatment ($P = < 0.05$). The value shown includes five experiments in which morphine produced no increase over baseline release.

**** $P = < 0.001$.

voked release of purine in six out of eleven experiments. In five experiments, however, morphine completely failed to induce purine release. Application of K^+ to the cerebral cortex evoked release comparable to that observed in preceding experiments. Because of a lack of consistency in the effect of morphine, the antagonistic action of naloxone against this agonist could not be tested with confidence and instead the action of both Leu-enkephalin and FK 33,824 on purine release was tested under similar conditions. Neither Leu-enkephalin nor FK 33,824 influenced the spontaneous release of purines from the cerebral cortex.

Discussion

In the present study both Theo and caffeine antagonized the inhibitory effect of Met-, Leu-enkephalin and morphine on the twitch height in the myenteric plexus longitudinal muscle preparation stimulated to produce submaximal response. This antagonism is apparent at a dose of methylxanthines (10^{-4} M) which does not affect the response of tissues to exogenous ACh (Sawynok & Jhamandas, 1976). The failure of some investigators to observe the antagonism of morphine action by Theo in similar tests could be attributable to the use of a lower concentration of the methylxanthine or the use of supramaximal stimulation of the methylxanthine or the use of supramaximal stimulation (Gallant & Clement, 1981; Vizi *et al.*, 1981). Indeed, in the present study Theo failed to antagonize the action of Met-enkephalin or morphine in tissues that were stimulated supramaximally. In contrast, caffeine antagonized the effect of both opioid agonists under this condition. The reason for the failure of Theo to act in maximally stimulated tissue is not known. It is possible that when supramaximal field stimulation is used to elicit the twitch, some of Theo added to the tissue bath undergoes electrolytic degradation (Nakatsu & Bartlett, 1979) and its concentration falls below the critical level required to antagonize the opioid action. Caffeine presumably undergoes less or no degradation. Under conditions of maximal stimulation Theo readily antagonizes the action of adenosine and adenine nucleotides on the MPLM preparation (Sawynok & Jhamandas, 1976; Gallant & Clement, 1981). This may be due to the fact that Theo is a more effective antagonist of adenosine, and that the fraction of the drug escaping degradation is sufficient to block adenosine receptors. Recently Brailowsky *et al.* (1981) have demonstrated that a dose of Theo even lower than that used in the present study antagonizes the action of morphine on the guinea-pig ileum preparation stimulated supramaximally. The use of a whole ileum preparation in that study, or a longer pretreatment period with Theo (30 min), could be a factor in the successful demonstration of antagonism.

Although experiments presented here demonstrate that methylxanthines can antagonize opioid action on the MPLM preparation under certain conditions, these do not constitute evidence for the proposal that this action is mediated by adenosine. A lack of adenosine deaminase action against morphine (Jhamandas & Sawynok, 1976), higher doses of methylxanthines required to antagonize morphine (Sawynok & Jhamandas, 1979) and a difference between the relative potency of Theo and caffeine when tested against morphine and adenosine (un-

published observations), argue against involvement of adenosine or its derivatives in the inhibitory action of opioids on the guinea-pig MPLM preparation.

The idea that adenosine mediates the inhibitory action of opioids on release of brain ACh is not supported by the results of purine release experiments performed here. Systemically administered morphine failed to release purines from the cerebral cortex prelabelled with a low [^{3}H]-adenosine concentration. Since it has been reported that morphine stimulates purine release from the cortex prelabelled with a relatively high concentration of adenosine (Phillis *et al.*, 1980), the effect of the agonist in these conditions was also tested in the present study. Morphine indeed induced purine release from the cortex, but this effect was not consistently observed. In nearly 50% of experiments performed under this prelabelling condition, morphine failed to raise purine release above baseline level, making it difficult to assess the specificity of its action in the naloxone antagonism experiments. Both Leu-enkephalin and FK 33,824, when tested at doses that clearly depressed ACh release, completely failed to increase the spontaneous purine release. The failure of enkephalins, or morphine in certain experiments, to exert a stimulatory effect is not attributable to disfunction of the tissue preparations since K^+ application consistently provoked purine release, and preparations that appeared to be poor in quality were specifically excluded from the study. Thus, to the extent that the efflux of radiolabelled purines reflects adenosine release, the activation of receptors that mediate the inhibitory effects of opioids on cortical ACh release does not always trigger a release of adenosine.

Morphine and enkephalins previously have been found to increase the stimulus-evoked but not the spontaneous release of radiolabelled purines from isolated cortical slices by a naloxone-sensitive mechanism (Fredholm & Vernet, 1978; Stone, 1981). However, neither morphine nor enkephalins exert an opiate receptor-mediated action on the spontaneous or the evoked release of ACh from cortical slices (Jhamandas *et al.*, 1975; Szerb, 1974; Jhamandas & Elliott, 1980). The studies performed so far indicate that morphine and enkephalins probably inhibit cortical ACh release by acting at a subcortical site. In view of these facts it would seem that the effects of opioids on purine release are independent of their action on cortical ACh release. However, this does not exclude the possibility that adenosine is involved in some other cortical actions of opioids (Perkins & Stone, 1980).

The mechanism underlying the methylxanthine antagonism of enkephalins or other opioids is not known at present. The fact that methylxanthines mobilize stores of calcium (Chapman & Miller, 1974), and that calcium antagonizes opiate-induced inhibition of ACh release in the periphery and in the brain (Jhamandas, Sawynok & Sutak, 1978; Opmeer & Van Ree, 1979; Sawynok & Jhamandas, 1979), suggests that these agents may exert their antagonistic action by preventing the depressant action of opioids on the entry of neuronal calcium (Guerrero-Muñoz, Cerrata, Guerrero & Way, 1979). This explanation derives some support from the observation that Theo blocks morphine-induced inhibition of ^{45}Ca uptake by brain synaptosomes (Brailowsky *et al.*, 1981).

Our observation that methylxanthines like Theo and caffeine antagonize a naturally occurring enkephalin may have implications for certain therapeutic uses of methylxanthines. The latter are now used in the treatment of certain apnoeas, especially the apnoea of premature infants (Arnanda & Turmen, 1979). It is interesting to speculate that the therapeutic benefit of methylxanthines in this regard is derived from the antagonism of opioids acting as endogenous respiratory depressants.

The authors acknowledge the generous gift of FK 33,824 from Dr D. Roemer (Sandoz, Basel, Switzerland). This work was supported by the Medical Research Council of Canada. H.N. is a recipient of student scholarship support from Huntington Society (Canada). We thank Dr W.J. Racz for helpful comments and Mrs Janet LeSarge and Mrs D. Browne for typing the manuscript. Correspondence to K.J. please.

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(Received June 2, 1983. Revised July 22, 1983.)