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THE EFFECTS OF MORPHINE AND METHIONINE-ENKEPHALIN ON THE RELEASE OF PURINES FROM CEREBRAL CORTEX SLICES OF RATS AND MICE

T.W. STONE

Dept. of Physiology, St. George's Hospital Medical School, University of London, London SW17 0RE

1 Slices of cerebral cortex from Wistar rats, TO mice or C57 mice were preincubated with [^{3}H]-adenosine, and labelled purines were subsequently released by electrical stimulation or by perfusing with ouabain, 100 μM.

2 Electrically-evoked purine release was substantially reduced when the Ca^{2+} concentration in the medium was lowered from 2.4 to 0.1 mM. In both rats and mice, the electrically-evoked release was increased by morphine and methionine-enkephalin (Met-enkephalin), 10 μM and in rats and TO mice by morphine 1 μM, both drug effects being prevented by naloxone.

3 Purine release evoked by ouabain was also increased by morphine 1 and 10 μM, though not by Met-enkephalin, from slices of rat cortex. Ouabain-induced release from TO mice was reduced by morphine, and from C57 mice was unchanged.

4 The enhancement by morphine of electrically-evoked purine release may indicate that purines mediate some effects of morphine in the CNS.

Introduction

A number of studies have led to the view that the inhibitory effects of morphine on neurotransmitter release or cell firing may involve the initial release of adenosine. Thus, both compounds are potent inhibitors of transmitter release (Ginsborg & Hirst, 1972, Henderson, Hughes & Kosterlitz, 1972; Henderson & Hughes, 1976; Ribeiro 1979; Harms, Wardeh & Mulder, 1979; Stone, 1981a,b) and the effects of both are prevented by adenosine antagonists such as theophylline (Gintzler & Musacchio, 1975; Sawynok & Jhamandas, 1976; Jhamandas, Sawynok & Sutak, 1978; Burnstock, 1978; Perkins & Stone, 1980a,b).

However, recent observations on the mouse vas deferens indicate that in this preparation at least, the inhibitory effects of morphine are not blocked by theophylline, implying that adenosine mediation is not always an essential feature of morphine's depression of transmitter release (Stone, 1981a).

Furthermore, a number of attempts have been made to seek opiate effects on purine release directly but these have tended to yield conflicting results. Fredholm & Vernet (1978) for example, found a small but significant enhancement by morphine of purine release evoked by veratridine from slices of cerebral cortex. Phillis, Jiang, Chelack & Wu (1979) subsequently demonstrated a naloxone-sensitive increase by morphine of purine release from the rat cerebral cortical surface *in vivo*.

Most recently, Jhamandas & Dumbrille (1980) re-examined purine release from the rat cerebral cortex surface *in vivo* but showed a reduction by morphine of glutamate-evoked purine release. The differences between the results of this group and of Phillis *et al.* (1979) may be explained by the use of different anaesthetics, male (Phillis *et al.*, 1979) versus female (Jhamandas & Dumbrille, 1980) rats, the use of glutamate as a stimulus, or the use of injections and topical applications of drugs, respectively.

Many of the previous functional studies implicating adenosine in the effects of morphine have involved electrical stimulation (Gintzler & Musacchio, 1975; Sawynok & Jhamandas, 1976) or spontaneous electrical activity (Perkins & Stone, 1980b). In the present study therefore, the effects of morphine and methionine-enkephalin (Met-enkephalin) have been examined on purine release evoked by electrical stimulation of slices of cerebral cortex of rats and of mice. Experiments were also performed on ouabain-evoked release for comparison.

Methods

Adult male Wistar rats, or male mice of either TO or C57/BL10 strains were killed by stunning and cervical dislocation. The brain was rapidly exposed and the dorsal lying areas of cerebral cortex removed into

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a solution at 0°C of the following composition (mM): NaCl 124, KCl 5, KH_2PO_4 1.24, $MgSO_4$ 1.3, $CaCl_2$. $2H_2O$ 2.4, $NaHCo_3$ 26 and glucose 10.

The cortex was then chopped perpendicularly to the pial surface into 400 μm sections, with a McIlwain tissue chopper. The slices were transferred into an incubation chamber containing the same medium as above at 37°C and saturated with 95% O_2 and 5% CO_2. After a 20 min pre-incubation period, 1 μCi of [^{3}H]-adenosine (sp. act. 20 Ci/mmol; 0.05 nmol; Amersham) was added to the chamber and incubation continued for a further 30 min. During the early experiments of this series, the contents of the chamber were subsequently removed and replaced completely every 2 min by gentle suction via taps at the base of the chamber. The viability of this system has been confirmed previously in a study of the potassium-evoked release of GABA (Hollins & Stone, 1980a). During this part of the experiment, the uptake of any released adenosine was prevented by the inclusion of dipyridamole 10^{-5}M in the bathing medium.

In later experiments, the slices were superfused continuously with oxygenated medium at 37°C, at a rate of approximately 0.35 ml/min after incubating with the labelled purine. Adenosine release was induced either by including ouabain (10^{-4}M) in the medium for a total of 20 min or by electrical stimulation. For the latter procedure, silver/silver chloride plates, approximately 4 × 2 mm were positioned immediately above and below the slices in the perfusion chamber. Stimuli were delivered at a frequency of 40 Hz, 1 ms duration, 50 V, and of alternating polarity (20 Hz each phase) for 2 min. The use of alternating polarity pulses and Ag/AgCl electrodes reduced problems of polarization, and when monitored on an oscilloscope, stimulating current remained unchanged for the period of stimulation.

After an initial washout period of 30 min, the bathing medium was collected in 2 min samples, and 0.5 ml aliquots mixed with a scintillation fluid (Fisofluor) and counted on a liquid scintillation counter (Kontron SL3000). Counting efficiency was about 42%.

At the end of each experiment the slices were themselves placed in scintillant, shaken vigorously and counted.

Results were plotted as the radioactivity per sample expressed as a fraction of that remaining in the tissue at the end of the experiment. The additional release produced by ouabain or electrical stimulation was taken as the increment of release between the peak of the release curve and the estimated baseline at that time, expressed as a percentage of the baseline.

Results were analysed by use of Student's *t* test, assuming a normal distribution of results.

In some experiments samples of perfusate were subjected to thin layer chromatography. Purines were first adsorbed onto activated charcoal, eluted into distilled water and these desalted solutions were then freeze-dried. After reconstituting into a small volume of water, 20 μl samples were applied to silica gel plates containing a fluorescent additive (Merck), allowed to dry and then run in a mixture of ethylacetate: *n*-butanol: methanol: ammonia (4:7:3:4 v/v) (Shimizu, Creveling & Daly, 1970). Purine spots were subsequently located under u.v. light, scraped into scintillation vials and counted. Counts were detected in the three areas corresponding to adenosine and its metabolites inosine and hypoxanthine in the approximate ratio 20:35:45.

Results

The Ca^{2+} dependency of ouabain-evoked release has been described previously (Hollins & Stone, 1980b). Complete removal of Ca^{2+} from the medium abolishes ouabain-evoked release but in 0.1 mM calcium, release is increased (Table 1). The effect of reducing the Ca^{2+} content of the perfusing medium

Table 1 The effect of low Ca^{2+}-containing media on purine release from brain slices evoked by electrical stimulation or by ouabain

			Release (% min^{-1})	
Stimulus	*Calcium* (mM)	*Wistar rat*	*TO mouse*	*C57 mouse*
None (spontaneous)	2.4	0.64 ± 0.04 (28)	0.73 ± 0.04 (32)	0.32 ± 0.04 (4)
None (spontaneous)	0.1	0.95 ± 0.11 (8)	0.94 ± 0.12 (4)	0.38 ± 0.04 (3)
Elec. Stim.	2.4	2.45 ± 0.16 (19)*	2.24 ± 0.10 (15)*	0.66 ± 0.05 (3)*
Elec. Stim.	0.1	1.26 ± 0.15 (5)	1.10 ± 0.08 (4)	0.53 ± 0.06 (3)
Ouabain (100 μM)	2.4	1.20 ± 0.05 (20)*	1.38 ± 0.04 (10)*	0.65 ± 0.05 (6)*
Ouabain (100 μM)	0.1	1.46 ± 0.12 (6)*	1.42 ± 0.08 (5)*	0.68 ± 0.05 (5)*

Peak release is expressed as a percentage of that remaining in the tissue at the end of the experiment, and results are shown as mean ± 1 s.e. (*n*).

*Significantly different from the spontaneous release at the same calcium concentration ($P < 0.02$, Student's *t* test).

Table 2 The effects of morphine and Met-enkephalin on ouabain (100 μM)-evoked release of ^{3}H-purines from cortical slices

Treatment	*Release increment (%)* *Wistar rat*	*TO Mouse*	*C57 mouse*
Control (ouabain alone)	87.5 ± 9.4 (20)	89.0 ± 11.4 (10)	103 ± 21.4 (6)
Morphine (1 μM)	132.6 ± 12.4 (8)*	79.2 ± 6.4 (6)	92.6 ± 8.5 (6)
Morphine (1 μM) + naloxone (0.1 μM)	84.8 ± 13.2 (4)**	93.5 ± 3.2 (8)	–
Naloxone (1 μM)	79.5 ± 13.1 (3)	71.3 ± 4.2 (3)	85.4 ± 8.3 (3)
Morphine (10 μM)	195.6 ± 15.5 (10)*	33.5 ± 4.6 (8)*	84.8 ± 12.2 (6)
Morphine (10 μM) + naloxone (0.1 μM)	123.3 ± 12.4 (5)**	58.6 ± 7.2 (4)**	–
Morphine (10 μM) + naloxone (1 μM)	81.4 ± 16.1 (4)**	87.6 ± 5.2 (7)**	–
Met-enkephalin (1 μM)	98.8 ± 10.9 (4)	–	–
Met-enkephalin (10 μM)	102.6 ± 19.4 (6)	96.8 ± 7.4 (4)	90.6 ± 6.4 (4)

For ease of comparison the peak increment of release produced by each treatment is expressed as a percentage increase over the estimated baseline. Data are shown as mean ± 1 s.e. (*n*).
*Significantly different from control values ($P<0.02$, Student's *t* test); **significantly different from values with morphine or Met-enkephalin alone ($P<0.02$).

to 0.1 mM on the electrically evoked release of purines is summarised in Table 1. There was a reduction of the electrically evoked release increment to about 17% of that in normal (Ca^{2+} 2.4 mM) media with rat slices and to 11% of normal with TO mouse slices. A small increase was noted in the level of baseline release in low Ca^{2+} media.

With tissue from Wistar rats, morphine at 1 and 10 μM caused a small but significant elevation of ouabain-evoked purine release (Table 2). At both concentrations this effect could be blocked by naloxone, 0.1 or 1 μM. Naloxone alone produced a small and non-significant reduction of release. Met-enkephalin, 1 or 10 μM induced no apparent change of the ouabain-evoked release.

In contrast, ouabain evoked release from TO mice was substantially reduced by morphine at 10 μM, an effect that was again prevented by naloxone. How-

Table 3 The effects of morphine and Met-enkephalin on electrically-evoked release of [^{3}H]-purines from cortical slices

Treatment	*Release increment (%)* *Wistar rat*	*TO mouse*	*C57 mouse*
Control (Stim. only)	283 ± 18.4 (5)	207 ± 13.2 (26)	106 ± 10.1 (3)
Morphine (1 μM)	362 ± 20.5 (5)*	286 ± 18.6 (6)*	142 ± 9.0 (4)
Morphine (1 μM) + naloxone (0.1 μM)	291 ± 15.2 (4)**	221 ± 11.4 (4)**	121 ± 10.9 (4)
Morphine (10 μM)	425 ± 19 (6)*	566 ± 26.9 (16)*	210 ± 13.8 (4)*
Morphine (10 μM) + naloxone (0.1 μM)	354 ± 22.7 (4)**	383 ± 16.2 (4)**	–
Morphine (10 μM) + naloxone (1 μM)	319 ± 16.2 (5)**	252 ± 20.1 (5)**	116 ± 18.5 (3)**
Met-enkephalin (1 μM)	322 ± 11.8 (4)	–	–
Met-enkephalin (10 μM)	399 ± 16.5 (4)*	421 ± 16.8 (5)*	314 ± 16.2 (4)*
Met-enkephalin (10 μM) + naloxone (0.1 μM)	342 ± 18.4 (4)	–	–
Met-enkephalin (10 μM) + naloxone (1 μM)	310 ± 19 (4)**	238 ± 14.7 (4)**	135 ± 12.6 (4)**
Dextrorphan (2 μM)	268 ± 23.2 (3)	251 ± 16.6 (3)	109 ± 20.0 (3)
Levorphanol (2 μM)	384 ± 16.9 (5)*	412 ± 22.5 (4)*	218 ± 13.5 (4)*

Details as for Table 1.
*Significantly different from control values ($P<0.05$, Student's *t* test); **significantly different from values with morphine or Met-enkephalin alone ($P<0.05$).

ever, with slices from C57/BL 10 mice, ouabain-evoked release was less in absolute terms (Table 1) than from the TO strain, and no significant effect of morphine on purine release could be detected (Table 2). Met-enkephalin was again inactive on tissue from either strain.

Electrically-evoked release yielded somewhat more consistent results, in that release from slices of all three tissue types was increased by morphine at 10 μM (Table 3), and was increased by morphine at 1 μM from rat and TO mouse slices. In the case of rat cortex, this action was still small, but was clearly significant and prevented by naloxone. Met-enkephalin was also active at 10 μM on tissue from all three strains (Table 3), and this effect was blocked by naloxone at the higher concentration of 1 μM.

Table 3 also shows that at concentrations of 2 μM levorphanol but not dextrorphan caused a significant increase of the electrically-evoked release.

Discussion

There has been a great deal of discussion recently about the validity of different methods for evoking neurotransmitter release. Thus the use of sine wave or alternating polarity stimuli has been advocated to avoid changes of temperature and tissue damage which may have accounted for the partial Ca^{2+}-dependency of transmitter release in early studies (Orrego, 1979; Fagg & Lane, 1979; Birsel & Szerb, 1980). At the same time, the use of low Ca^{2+} solutions has been preferred to Ca^{2+}-free media for testing Ca^{2+}-dependency as the Ca^{2+}-free solutions encourage a sodium-dependent release mechanism, probably involving intracellular Ca^{2+} release, leading to an underestimation of Ca^{2+}-dependency (Valdes & Orrego, 1978; Sandoval, 1980). The substantial Ca^{2+}-dependency of electrically-evoked release in the present experiments revealed by use of low Ca^{2+} solutions is probably therefore a meaningful reflection of a requirement for external Ca^{2+} (McIlwain, 1972). As electrically -evoked release from damaged tissues, or K^+-evoked release from normal tissues is largely independent of Ca^{2+} (Pull & McIlwain, 1973; Birsel & Szerb, 1980; Stone, 1981b) this also implies that we are observing a Na^+/Ca^{2+} mediated release from undamaged tissue which should therefore approximate to any electrically related physiological release mechanism.

It is from this point of view that the enhancement by morphine and Met-enkephalin of evoked purine release from rat cerebral cortex becomes especially interesting. The previous functional studies which have led to the idea that some effects of morphine may involve an initial release of adenosine have been based upon electrical activity, either resulting from artificial stimulation (Gintzler & Musacchio, 1975; Sawynok & Jhamandas, 1976) or from spontaneous activity (Perkins & Stone, 1980b) so that the enhancement by morphine of electrically induced release from tissues is at least consistent with the earlier work. The previously noted enhancement of veratridine-mediated release (Fredholm & Vernet, 1978) and the present increase of ouabain-induced release from rat tissue, support the suggestion that purine release from brain slices involves the influx of Na^+ and Ca^{2+} ions (Hollins & Stone, 1980b), the movement of which is of course physiologically coupled by the action potential mechanisms.

In general, Met-enkephalin appears to be less effective than morphine in changing purine release. It is tempting to relate this to the suggestion that opiate actions on purine release may involve μ-receptors rather than δ-receptors (Stone, 1981a), as morphine is a more effective agonist than Met-enkephalin at μ-receptors (Lord, Waterfield, Hughes & Kosterlitz, 1977). However, it is likely that Met-enkephalin is metabolized rapidly by the brain slices and this could well account for its relatively weak actions. It should also be borne in mind that Met-enkephalin may be degraded or inactivated more rapidly when electrical stimulation is being used (Kitchen & Hart, 1981), so that the present experiments may be providing an underestimate of the potency of Met-enkephalin.

With respect to the tissue from mice used here, it is clear that morphine has less effect on the C57 slices than the TO slices. It is therefore interesting to recall that Henderson & Hughes (1976) have previously noted the marked insensitivity to morphine of the field-stimulated isolated vas deferens of the C57 strain. It is possible then that the C57 mice lack opiate receptors or receptor-associated processes at least for the classical agonist morphine, and that whatever the nature of deficiency in the vas deferens, a comparable deficiency exists in the CNS.

The observation for which there is at present no convincing explanation is the reduction of ouabain-evoked purine release from TO mouse cortex. However, previous examination of the characteristics of ouabain-evoked purine release was performed on tissue from rats only (Hollins & Stone 1980b) and it is possible that the properties of cortex slices important for ouabain-evoked release differ in rats and mice. We have concluded elsewhere that purine release by ouabain is probably not due to a simple inhibition of (Na^+, K^+) ATPase (Hollins, Stone & Lloyd, 1980; Stone, Hollins & Lloyd, 1981; Lloyd & Stone, 1981).

Thus morphine can enhance the electrically-evoked release of purines from cortical slices, a finding consistent with the experiments of Fredholm & Vernet (1978) and Phillis *et al.* (1979). These results are at variance with the work of Jhamandas & Dumbrille (1980), but in the latter work, glutamate was

used routinely as the releasing stimulus. As morphine has been shown on a number of occasions to reduce glutamate depolarization directly, as the authors themselves note (also Perkins & Stone 1980b) the failure of morphine to enhance glutamate-evoked purine release is less surprising.

It is difficult to estimate the relevance of morphine-purine interactions for the behavioural effects of the opiate. Since adenosine and morphine both inhibit transmitter release from the rat striatum (Celsen & Kuschinsky, 1974; Loh, Brase, Sampath-Khanna, Mar & Way, 1976; Michaelis, Michaelis & Myers, 1979) it is conceivable for example, that a release of adenosine could be involved in the suppression by morphine of dopamine turnover, locomotor activity and turning behaviour in rats (Kuschinsky & Hornykiewicz, 1972). The possibility also requires serious consideration that the quasi-morphine abstinence syndrome produced by methylxanthines (Collier, Francis, Henderson & Schneider, 1974; Francis, Roy & Collier, 1975) results, at least in part, from the ability of these compounds to block the effects of endogenous adenosine (Burnstock, 1978; Perkins & Stone 1980a; Stone 1981b).

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