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Relationship between morphine analgesia and cortical extracellular fluid levels of morphine and its metabolites in the rat: a microdialysis study

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1 The effect of morphine (10 mg kg^{-1}, s.c.) on the analgesic response measured by the tail-flick method was determined in male Sprague-Dawley rats. The analgesic response to morphine was correlated with the levels of morphine and its metabolites collected by microdialysis from the cortical extracellular fluid (ECF).

2 The analgesic response to morphine lasted for 4 h. The concentration of morphine during a 4 h collection period was significantly higher than the metabolites concentration. The relative concentration of morphine and its metabolites during the 4 h period was 70 and 30% respectively.

3 The analgesic response during the first 2.25 h period accounted for more than 82% of the total analgesia as determined by the area under the time-response curve (AUC). The concentration of morphine and its metabolites during the same period were 78 and 22%, respectively, but they did not differ during the 2.25-4.0 h period (52 and 48%).

4 The half-life for morphine and its metabolites were similar, the maximal achievable concentration C_{max} and $AUC_{0-4\,h}$ were lower for metabolites but the time to reach maximum concentration was higher for morphine metabolites than for morphine. The ratio of the concentration of metabolites to the concentration of morphine in the cortical ECF increased with time whereas the analgesic response to morphine decreased with time.

5 At several time points following morphine injection even though the levels of morphine were the same, the concentration of metabolites (mainly M3G) differed and thus the ratio [metabolite/morphine]. A plot of [metabolite]/[morphine] vs. analgesia gave a high correlation coefficient. Since M3G has been shown to be antianalgesic and is the only metabolite of morphine in the rat, it is concluded that the levels of this metabolite may regulate the analgesic effect of morphine in the rat.

Keywords: Morphine metabolites; M3G; microdialysis; cortical extracellular fluid; analgesia

Introduction

Morphine is an opiate which is commonly used to alleviate pain of moderate to severe intensity. The mechanism by which morphine produces its action is far from clear. In recent years, considerable attention has been paid to the potential role of metabolites of morphine in the elicitation of pharmacological responses. The metabolic fate of morphine depends on the species studied. In man it is metabolized to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) (Yeh *et al.*, 1977) in the liver and kidneys (Pacifi & Rane, 1982; Wahlstrom *et al.*, 1988). The glucuronide formation can also occur in the microsomal fraction of the rodent brain (Wahlstrom *et al.*, 1989) as well as of the human brain (Wahlstrom *et al.*, 1988). In man and rats the formation of glucuronides of morphine is enantioselective and regioselective. The glucuronidation of morphine in the two species appears to require the involvement of two UDP-glucuronosyltransferases. In rats, the natural (-)-morphine is metabolized to (-)-M3G only, but the unnatural (+)-morphine is transformed principally to (+)-M6G and to (+)-M3G as the minor metabolite. In man, (-)-morphine is converted to (-)-M3G, as the major metabolite and (-)-M6G as the minor metabolite. On the other hand, (+)-morphine is metabolized predominantly to (+)-M6G and to a lesser amount to (+)-M3G in human liver microsomes (Coughtrie *et al.*, 1989).

The pharmacological actions of M3G and M6G have been studied. M6G appears to be active and more potent than morphine in several systems. Thus, it has been shown to produce analgesia in man (Osborne *et al.*, 1988; Hanna *et al.*, 1990) and in rodent (Shimomura *et al.*, 1971; Yoshimura *et al.*, 1973; Pasternak *et al.*, 1987; Abbott & Palmour *et al.*, 1988; Paul *et al.*, 1989; Sullivan *et al.*, 1989; Gong *et al.*, 1991; 1992; Frances *et al.*, 1992). It also possesses a respiratory depressant action in guinea-pigs (Murphey & Olsen, 1994).

While M3G was initially thought to be pharmacologically inactive, several recent studies have suggested it to have antianalgesic and hyperalgesic effects. It antagonizes the analgesic (Smith *et al.*, 1990) and ventilatory (Gong *et al.*, 1991; 1992) effects of morphine as well as of M6G, and appears to have long biological half-life (Ekblom *et al.*, 1993). Because of these observations, it has been suggested that the ratio M6G/M3G in serum and/or CSF may be important in the overall analgesic activity of morphine (Bowsher, 1993).

Since in the rat (-)-morphine is metabolized only to M3G (Coughtrie *et al.*, 1989; Kuo *et al.*, 1991), the present studies were carried out to determine the role of morphine metabolites in the analgesic response to morphine. The concentration of morphine and its metabolites were determined by radioimmunoassay in the dialysate which reflect the activity in the cortical extracellular fluid (ECF). The dialysate was collected by using transcortical microdialysis procedure.

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Methods

Animals

Male Sprague-Dawley rats weighing 225–250 g obtained from Taconic Farms, Germantown, NY or Charles River, France, were acclimatized to a room with controlled ambient temperature (23 ± 1°C), humidity (50 ± 10%) and a 12 h dark-light cycle (light 06 h 00 min–18 h 00 min). The animals were housed under these conditions for at least 4 days before being used. The rats were given food and water *ad libitum*.

Measurement of the analgesic response

The analgesic response to morphine (10 mg kg^{-1}, s.c.) was determined by using a tail-flick apparatus as described previously (Bhargava *et al.*, 1987). The tail-flick latencies to thermal stimulation were determined before and at various times up to 240 min after injection of the drug. The basal latency was found to be approximately 2 s. A cut-off time of 10 s was used to prevent any injury to the tail. The basal response was subtracted from the effect induced by morphine. The analgesic response was expressed as % maximal possible effect (MPE) and calculated by the formula $\%MPE = [T_t - T_0)/(10 - T_0)] \times 100$, where T_0 and T_t are the tail-flick latencies before and after a specific time following the administration of morphine.

Pharmacokinetics of morphine in cortical ECF

In vivo *microdialysis* The microdialysis probe was an acrylonitrile-sodium methallyl sulphonate membrane (AN69 HF Filtral 12; inner diameter (damp) 240 μm; outer diameter 290 μm; molecular weight cutoff 44,000; Hospal Industry S.A., 69883 Meyzieu, France). The dialysis tubing was coated with epoxy glue, except for a 8 mm dialysis tip corresponding to the length of the rat parietal cortex. The procedure used to insert the transcortical dialysis fibre was essentially the same as that described by Barjavel *et al.* (1994). Rats (Charles River, France) were anaesthetized with chloral hydrate (300 mg kg^{-1}, i.p.) and placed in a stereotaxic frame (David Korpf Instruments Roucaire, Velizy Villacoublay, France). To implant the transcortical probe, two holes were drilled on the side of the skull (coordinates according to Paxinos & Watson, 1986, respectively from bregma: A-P, 0.3 mm, V 2.0 mm). The dialysis fibre, held straight by an internal tungsten wire was implanted transversely in the cerebral cortex. The tungsten wire was withdrawn and two stainless steel tubes (23 gauge needle/10 mm long) were glued to the end of the fibre to form the outlet (Di Chiara *et al.*, 1990; Barjavel *et al.*, 1994). After surgery, rats were allowed to recover overnight. Rats were supplied with food and water *ad libitum*.

Dialysis experiments were performed 24 h after probe implantation for total blood-brain-recovery (Barjavel *et al.*, 1994) by perfusing the dialysis probe with Ringer solution (NaCl 147 mM, KCl 4 mM, $CaCl_2$ 2.4 mM, pH adjusted to 7.3 with NaOH and controlled before and after the experiments) at a constant flow rate (3 μl min^{-1}), by a microinfusion pump (Precidor, Infors AG, Basel, Swiss). Samples for basal levels were collected. Rats were injected s.c. with morphine (expressed as base). The dialysate samples were continuously collected and fractionated every 30 min for 4 h in small vials and kept frozen until assays were performed.

Assays

Morphine was measured by a radioimmunoassay by using antibodies raised in goats against N-carboxymethylnormorphine (N-CMN) linked to bovine serum albumin (BSA). Antibodies were specific to morphine and exhibited no cross-reactivity (< 0.2%) with M3G and opiate peptides (< 0.05%) as previously described (Sandouk *et al.*, 1991). The total concentration of morphine plus metabolites was measured by using an antiserum raised in rabbits with 6-hemisuccinylmorphine (6-HSM) linked to BSA as an immunogen. This antibody exhibited 100% of cross-reactivity with morphine and M6G and 80% with M3G (Table 1). The metabolite concentration was determined by subtraction in each dialysate of the total amount of opiates and the morphine only. Since M3G is the only major metabolite of morphine in the rat, the contribution of the other metabolites would be less likely.

Determination of phamacokinetic parameters

The mid point of the 30 min collection periods was used in ECF concentration-time course as suggested by Stahl (1992). Maximal concentration extrapolated to time zero (C_{max}) and time for achieving maximal concentration (T_{max}) were observed values in brain cortical dialysates. Terminal half-life ($t_{1/2\beta}$) was determined from the terminal rate constant, β, calculated by linear regression analysis from the last 5 points by use of the Siphar program (Simed, Créteil, France). Area under the time concentration curve $(AUC)_{0-t}$ (zero to last concentration at time t) was calculated by the trapezoidal method from the experimental curve.

Drugs

Morphine sulphate was purchased from Mallinckrodt Chemical Co., St Louis, MO. The drugs was dissolved in saline and injected s.c. in a volume of 1 ml kg^{-1} body weight.

Statistics

Comparisons of pharmacokinetic parameters for morphine and its metabolites were performed by using Student's t test. A value of $P < 0.05$ was considered to be significant.

Results

Analgesic effects of morphine in the rat

The effect of morphine on the % MPE at various times after its administration is shown in Figure 1. A maximal response was observed for the first 1.5 h. At 2 h, the response was only 55% and at 4 h it was 15%.

Time course of the concentrations of morphine in cortical ECF

The plot of the concentrations of morphine and its metabolites with time following the administration of morphine (10 mg kg^{-1}, s.c.) to the rat is shown in Figure 2. The levels of morphine were significantly higher than those of morphine metabolites until 1.75 h but did not differ for the period of 2.25–4 h.

Table 1 % cross-reactivity of the two antisera with different opiates

	Antiserum *N-CMN*	*6-HSM*
Morphine	100	100
M3G	< 0.2	80
M6G	< 0.2	100
Normorphine	30	< 0.2
Codeine	< 0.2	84
β-endorphin	< 0.05	< 0.05
Leu-enkephalin	< 0.05	< 0.05
Met-enkephalin	< 0.05	< 0.05

N-CMN: N-carboxymethylnormorphine; 6-HSM: 6-hemisuccinylmorphine

Pharmacokinetics of morphine and its metabolites in cortical ECF

The pharmacokinetic parameters for morphine and its metabolites in cortical ECF of the rat are shown in Table 2. The C_{max} and $AUC_{0-4\,h}$ values for morphine were higher than for its metabolites. The $t_{1/2\beta}$ for morphine and its metabolites did not differ. The T_{max} values for morphine were significantly lower than for its metabolites (Table 2). The $AUC_{0-4\,h}$ for morphine and its metabolites were 70 and 30%, respectively.

The analgesic response during the first 2.25 h period was ≥82% of the total analgesia as determined by the area under the time-effect response curve. The concentration of morphine and its metabolites in the dialysates from the cortical ECF during the same period were 78 and 22%, respectively (Figure 3). The *in vitro* recovery of morphine and its metabolites (M3G and M6G) from the dialysate is shown in Figure 4. The recovery did not depend on either the opiate or the concentration of opiate used and was approximately 23–25% on average.

The time course of metabolites to morphine ratio in cortical ECF after administration of morphine to the rat is shown in Figure 5. The relative concentration of metabolites was only 13, 20, 32 and 60% that of morphine at 0.25, 0.75, 1.25 and 1.75 h after morphine injection. The ratio reached 1.0 at 2.25 h and remained so until 4 h observation period (Figure 5).

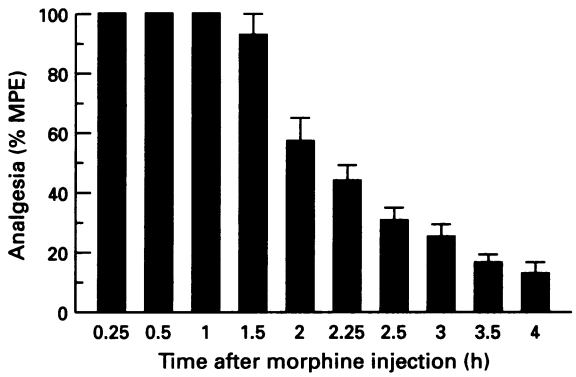

Figure 1 Time course of the analgesic effect of morphine (10 mg kg^{-1}; s.c.) in the rat. Tail-flick latencies were determined at various times after the injection of morphine to the rats. The response was expressed as % maximum possible effect (MPE) (means ± s.e.mean). Six rats were used for the study.

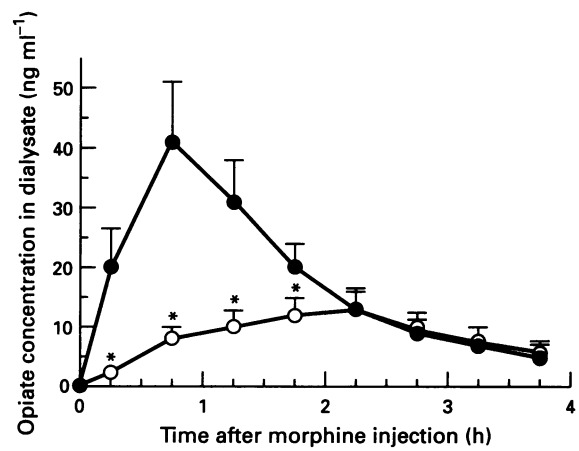

Figure 2 Time-course of the concentration of morphine (●) and its metabolites (○) in the cortical extracellular fluid (ECF) following the administration of morphine (10 mg kg^{-1}; s.c.) to the rat. The dialysate was collected at various times after morphine injection by use of microdialysis procedure. Morphine or its metabolite concentrations in the dialysate were determined by radioimmunoassay as described in the Methods section. Results are presented as means ± s.e.mean for six experiments. * Denotes significant differences ($P<0.05$) from the levels of morphine.

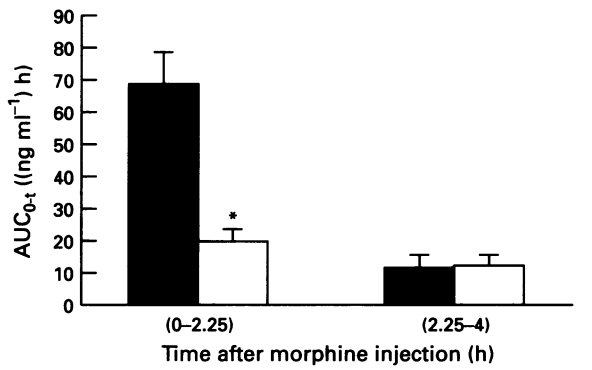

Figure 3 Concentration of morphine (■) and its metabolites (□) expressed as area under the time concentration cure (AUC) during the two time intervals (0.0–2.25 h) and (2.25–4.0 h) after morphine administration to the rat. Results are means ± s.e.mean of six experiments. * Denotes $P<0.05$ versus the concentrations of morphine.

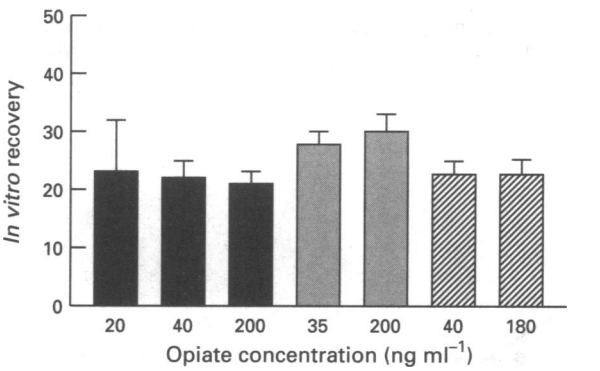

Figure 4 *In vitro* recovery of morphine and its metabolites at different concentrations of morphine-6-glucuronide (M6G, solid columns), morphine (hatched columns) and M3G (open columns). Results are means ± s.e.mean for six experiments.

Table 2 Pharmacokinetic parameters for morphine and its metabolites

	T_{max} (h)	$t_{1/2\beta}$ (h)	C_{max} (ng ml^{-1})	$AUC_{0-4\,h}$ (ng ml^{-1}) h
Morphine	0.75 ± 0.20	0.9 ± 0.2	36 ± 7	81 ± 11
Morphine metabolites	2.25 ± 0.25*	1.0 ± 0.2	15 ± 4*	33 ± 6*

Data shown are means ± s.e.mean.
*$P<0.05$ vs. morphine.

Figure 6 shows a plot of the ratio [metabolite/morphine] vs. the analgesic response at different times after the administration of morphine. The plot gave a straight line with a correlation of 0.975 which was highly significant with a P value of 0.0001.

Discussion

The present studies were conducted to assess the possible role of morphine metabolites in the analgesic action of morphine in the rat. Several studies indicate that in the rat, the major metabolite of morphine is the 3-glucuronide (Kuo *et al.*, 1991). Attempts were made to determine systematically the time course of the analgesic response to morphine and under the same conditions determine the levels of morphine and its glucuronide metabolites in the cortical ECF.

The analgesic response to morphine was maximal for a period of 1.5 h, started to decrease at 2.0 h and then declined gradually for the rest of the observation period. The concentration of morphine in the cortical ECF was maximal at 0.75 h and then declined exponentially. On the other hand, the concentration of morphine metabolite in the cortical ECF increased gradually with a maximal effect seen at 2.25 h. From 2.25 to 4 h period, the concentration of morphine and its metabolites were similar. A similar recovery of morphine (23%) at a flow rate of 2 μl min^{-1} has been observed previously (Matos *et al.*, 1992). In the present study, at a flow rate of 3 μl min^{-1}, the recovery of morphine as well as its metabolites was similar for the same dialysis membrane. Therefore, the flow rate did not appear to influence the recovery of morphine and its metabolites. Additionally, the recovery of morphine and its metabolites did not change during the 4 h observation period. Interestingly, the concentrations of morphine at 0.25 and 1.75 h were similar, yet the analgesic response was only 75% at 1.75 h and 50% at 2 h compared to that seen at 0.25 h. The difference was in the concentration of morphine metabolite and thus in the ratio of metabolite to morphine which was 0.13 and 0.60, respectively. This observation suggests that the levels of morphine metabolite may play a role in the diffential analgesic response to morphine at two different time points when the morphine concentrations are similar. Similarly, when the metabolites/morphine ratio reached a value of 1.0 and 2.25 h and remained the same until 4.0 h period, the analgesic response also did not change. When the ratio [metabolite]/[morphine] was plotted against the analgesic response at different times after the administration of morphine, and linear regression analysis was conducted, the regression line showed a correlation coefficient $r = -0.9875$ and a $P < 0.0001$ (Figure 6). This further supports our contention that the levels of the metabolite (M3G) may be regulating the levels of morphine-induced analgesia in the rat. Thus, morphine metabolites may have an antagonistic effect on morphine analgesia.

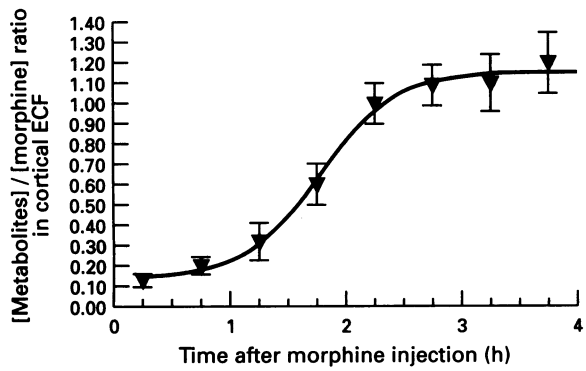

Figure 5 The ratio of metabolites to morphine concentration with time after the injection of morphine to the rat. The dialysate was collected at various times after the injection of morphine by use of the microdialysis procedure as described in the Methods section. The concentrations of morphine and its metabolites were determined by radioimmunoassay. Results are means ± s.e.mean of six experiments.

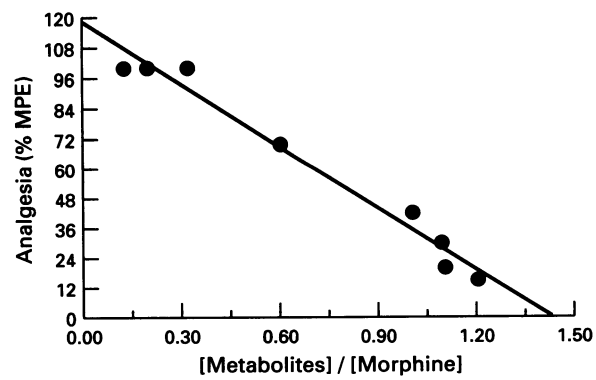

Figure 6 A plot of [metabolite]/[morphine] vs. the analgesic response (expressed as % maximum possible effect, MPE) at different times after the injection of morphine to the rat. The plot yielded a straight line with a negative slope and a high correlation coefficient ($r = 0.9875$) with $P < 0.0001$.

Intracerebroventricular (i.c.v.) or intrathecal (i.t.) administration of M3G has been shown to antagonize M6G-induced analgesia when the latter was also given by the i.c.v. route in the rat (Gong *et al.*, 1992). As indicated earlier, although M6G is not a metabolite of morphine in the rat, it is an active metabolite of morphine in several species including man (Schneider *et al.*, 1992; Goucke *et al.*, 1994). M3G has also been shown to be a potent antagonist of morphine-induced analgesia in rats (Smith *et al.*, 1990). Other studies indicate that M3G may be a functional antagonist of morphine or its active metabolite, M6G. Whereas morphine and its metabolite, M6G decreased both tidal volume and respiratory frequency, the hyperventilation induced by M3G was slightly potentiated by naloxone (Gong *et al.*, 1991). Although M3G has been demonstrated to be antagonistic to morphine- or M6G-induced analgesic and ventilatory effect in the rat (Gong *et al.*, 1992), the exact mechanisms of action remain obscure.

Several studies have shown that M3G causes non-opioid receptor mediated hyperalgesia (Labella *et al.*, 1979; Woolf, 1981). M3G does not appear to compete for opioid binding sites in the brain (Pasternak *et al.*, 1987; Paul *et al.*, 1989). Some studies indicate that its action may be due to interference with glycine (Curtis & Duggan, 1969; Woolf, 1981) or γ-aminobutyric acid (GABA) transmission (Robinson & Deadwyler, 1980).

Both M3G and M6G are found in plasma and cerebrospinal fluid after oral or intramuscular (Hand *et al.*, 1987; Poulain *et al.*, 1990) as well as after i.t. (Bigler *et al.*, 1990) administration of morphine to man. It has been suggested that after oral or intramuscular administration of morphine, up to 85% of its analgesic activity might be derived from M6G (Hand *et al.*, 1987). However, since in the rat, M6G is not a metabolite of morphine, the analgesic activity is primarily due to the concentrations of morphine and any change in the analgesic response with time has to be related to the levels of morphine and M3G.

Previously it was thought that the glucuronide metabolites are quite polar and pharmacologically inert. However, both M6G and M3G have been demonstrated to be quite lipid soluble and are able to penetrate the brain from the periphery

(Carrupt *et al.*, 1991; Yoshimura *et al.*, 1993). Thus, in the rat, after its formation in the periphery, M3G can easily penetrate the central sites of the rat to produce antagonism of the action of morphine. Studies in our laboratory have provided direct evidence that the two glucuronides of morphine can penetrate into and disappear from the brain with kinetics similar to those of morphine in the rat (Barjavel *et al.*, 1994).

In summary, the present studies have demonstrated a relationship between the levels of analgesia and the levels of morphine and its metabolites in the cortical ECF. At two different time points, even though the levels of morphine were similar, a significant difference in the analgesic response was obtained. The difference was, however, related to the higher levels of metabolites in the cortical ECF. Additionally, a high correlation coefficient for the plot of [metabolite]/[morphine] vs. the analgesic response further strengthens our view that in the rat, the metabolites of morphine may be regulating its analgesic action. Since M3G is the only major metabolite of morphine in the rat, the decreased analgesic response to morphine with time may be related to the higher level of this metabolite in the central nervous system. As indicated before, M3G appears to have an antagonistic action on opiates.

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