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INVOLVEMENT OF THE MEDIAN RAPHE NUCLEUS IN ANTINOCICEPTION INDUCED BY MORPHINE, BUPRENORPHINE AND TILIDINE IN THE RAT

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1 Antinociception induced by three analgesics with differing profiles of activity, morphine, buprenorphine and tilidine, have been evaluated in the hot plate and paw pressure tests after administration by the subcutaneous route and directly into the median raphe nucleus in the conscious rat.

2 Behavioural and neurological effects of the three analgesics were also assessed.

3 The typical profiles of antinociceptive activity induced by the three analgesics were qualitatively similar after either route of administration. Morphine induced naloxone-sensitive dose-dependent effects in both tests. Buprenorphine showed naloxone-sensitive effects with a bell-shaped dose-response curve in the thermal test but dose-dependent activity in the pressure test. Tilidine induced naloxone-sensitive dose-dependent effects in the thermal test but demonstrated naloxone-insensitive activity in the paw pressure test.

4 The behavioural and neurological effects of the analgesics in the dose range used would not have affected the animals' ability to respond to the nociceptive stimuli.

5 The results suggest that the median raphe could participate in drug-induced antinociception. The mechanisms by which this might occur are discussed.

Introduction

The role of 5-hydroxytryptaminergic function in both stimulation produced analgesia (SPA) and morphine activity has been extensively investigated. Messing & Lytle (1977) reviewed the literature and whilst there are discrepancies, the integrity of 5-hydroxytryptaminergic (5-HT) function seems intimately related to both stimulation-produced analgesia and opiate activity. Since almost all the 5-HT containing neurones in the brain and spinal cord originate from the raphe nuclei (Dahlström & Fuxe, 1964; Fuxe & Jonsson, 1974) it is of interest to explore the role of the raphe nuclei in central mechanisms of antinociception.

Fields & Basbaum (1978) have postulated a descending inhibitory mechanism by which SPA and the supra-spinal actions of morphine effect transmission of nociceptive information in the dorsal horn. 5-HT fibres originating in nucleus raphe magnus (NRM) and descending in the dorsal horn of the spinal cord have been implicated as part of the anatomical substrate for this system.

Evidence for the involvement of the mid-brain raphe nuclei in central mechanisms of analgesia, however, is conflicting (Cowan, Ghezzi & Samanin, 1974; Adler, Kostowski, Recchia & Samanin, 1975; Yaksh, Yeung & Rudy, 1976; Oleson, Twombly & Liebeskind, 1978; Oliveras, Guilbaud & Besson, 1979b).

To investigate further the role of the median raphe nucleus (MR) in drug-induced antinociception, three narcotic analgesics with differing profiles of activity, morphine, buprenorphine and tilidine (Herman, Steinbrecher & Heldt, 1970), were selected for evaluation following both subcutaneous administration and micro-injection into the MR. Preliminary results of this work (Bryant, Olley, Tyers & Marriott, 1978) were presented to the International Narcotic Research Conference, Noordwijkerhout, (1978).

Methods

Analgesic drugs were administered to Sprague-Dawley CFE male rats (270–340 g) either subcutaneously or directly into the MR through permanently implanted guide cannulae. Cannulae were inserted under anaesthesia (chloral hydrate 300–400 mg/kg) with the rat mounted in a stereotaxic frame with incisor bar raised 5 mm above the inter-aural line. The stainless steel cannula (11.5 mm

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long, 0.65 mm o.d., 0.3 mm i.d., fixed into a drilled tapped perspex holder so that 8.0 mm protruded) was screwed onto a cannula carrier which was tilted back through 22.5° to avoid the confluence of sinuses. Penetration at a midline point, marked by a small hole 2–3 mm caudal to lambda, and lowering the tip by 7.0 mm resulted in 90% successful accurate placements. The guide cannula was anchored with acrylic cement and its lumen occluded with a stainless steel stylet. Animals were used on one occasion only, at least seven days after operation.

Micro-injection into the MR in the conscious rat under gentle restraint was made with an Agla micrometer syringe and stainless steel injection tubing (0.3 mm o.d.) inserted 1 mm beyond the guide cannula tip. The dose volume, 1 μl, was delivered over 30 s, then after a further 60 s, the injection unit was withdrawn and the stylet replaced. At the completion of the experiment, 0.5 μl of 5% haemotoxylin was micro-injected into the anaesthetized rat and the site verified with reference to the stereotaxic atlas of Pellegrino, Pellegrino & Cushman (1979).

Antinociceptive tests

Antinociceptive activities were determined by use of the hot plate (Woolfe & MacDonald, 1944) and paw pressure (Randall & Selitto, 1957) tests.

In the hot plate tests, rats were placed on a copper plate (55 ± 1°C) and the latency to the hind paw lick response was recorded. Animals were removed immediately on responding or at 60 s if not responding. In the same animals and immediately following exposure to the hot plate the nociceptive pressure thresholds for both hind paws (non-inflamed) were determined using an Analgesymeter (Ugo Basile, Milan). The sequence of testing was always hot plate followed by paw pressure tests. Pressure was not allowed to exceed 500 g but responses of either vocalization or vigorous withdrawal of the limb terminated the test immediately.

Experimental design of subcutaneous administration studies

Nociceptive thermal and pressure thresholds were determined sequentially 3 h 30 min before and again at 30 min after drug administration. Morphine 0.03–10.0 mg/kg, burprenorphine 0.001–10.0 mg/kg and tilidine 5.0–80.0 mg/kg were administered in saline in a dose volume of 1.0 ml/kg. In each experiment 15 drug and 3 saline-treated animals were evaluated by a blind technique such that the operator was unaware of the drug treatments. Experiments were replicated and the data pooled for statistical analyses. Final dose-groups contained 9–12 animals.

The effects of naloxone 1 mg/kg or saline intraperitoneally, 15 min before the administration of analgesic were determined. Eight treatment combinations were assessed: naloxone or saline pretreatment followed by saline or single submaximal subcutaneous doses of morphine 0.3 mg/kg, buprenorphine 0.1 mg/kg or tilidine 20.0 mg/kg. Four of the treatments were assessed blind at each session and pooled results for each treatment ($n = 7$ or 8) were evaluated statistically. Observed behavioural effects of morphine 1–10 mg/kg, burprenorphine 0.1–10.0 mg/kg and tilidine 20.0–80.0 mg/kg were evaluated in separate experiments in which 3 drug- and 3 saline-treated animals were assessed at 30 and 60 min and thereafter every hour up to 5 h following injection.

Experimental design of median raphe administration studies

Antinociception and other behavioural effects were evaluated in the same treatment. Nociceptive thresholds were determined 4 h before and 5, 10, 20 and 40 min after drug administration. Animals were used once only. In each session 2 vehicle- and 4 drug-treated animals were evaluated blind, using healthy cannulated rats. Data were pooled and analysed for treatment groups containing 5 rats.

Naloxone sensitivity was tested by administering naloxone, 1 mg/kg, or saline intraperitoneally 15 min before saline, morphine 3.0 μg/rat, buprenorphine 0.1 μg/rat or tilidine 20 μg/rat. In each of these experimental sessions, 8 animals were evaluated blind. Nociceptive thresholds were determined sequentially 4 h before and again at 5, 10, 20 and 40 min after analgesic administration. The results of 6 such experiments were pooled.

Data analysis

Means and standard errors were calculated for all antinociceptive tests; levels of significance were determined by Student's *t* test. Regression analysis was used to determine linearity and parallelism of dose-response curves. 'Equi-effective antinociceptive doses' (EAD) were defined for hot plate and paw pressure tests respectively as 'the dose increasing the response latency to twice the mean pretreatment value' and 'the dose raising the nociceptive pressure threshold 100 g above pretreatment values'.

Drugs

Morphine sulphate (BDH), tilidine hydrochloride (Godecke AG), naloxone hydrochloride (Endo Laboratories) and choral hydrate (BDH) were dissolved in normal saline solution. Buprenorphine

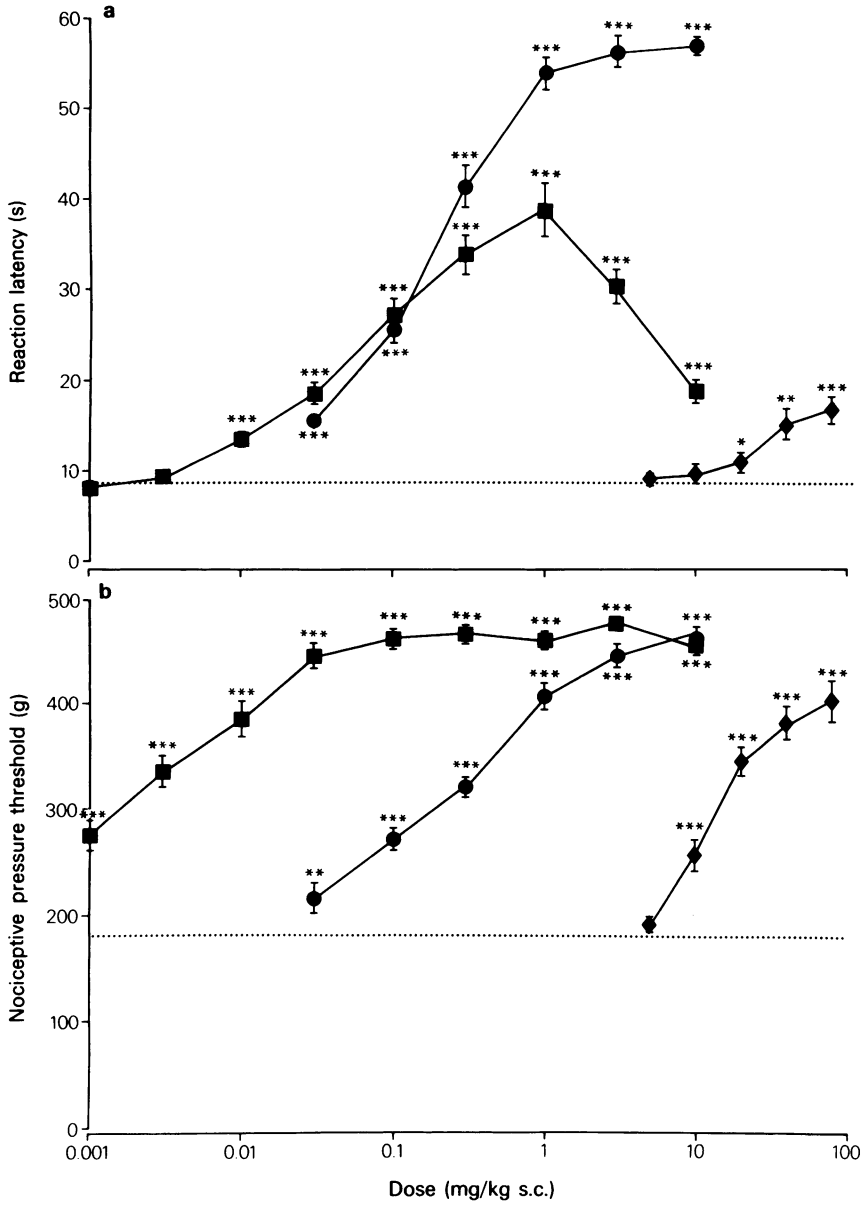

Figure 1 Antinociceptive activity was assessed in the hot plate test (a) and paw pressure test (b) 30 min after subcutaneous administration of morphine (●); buprenorphine (■) and tilidine (♦). Each point represents the mean post-drug nociceptive threshold from 12 or 9 animals. Vertical lines indicate s.e.mean. The broken lines are pre-drug mean nociceptive thresholds. Statistical comparison of pre- and post-drug threshold (Student's *t* test): $*P<0.05$; $**P<0.01$; $***P<0.001$.

hydrochloride (Reckitt and Colman Ltd) was diluted from the ampoule (1 mg/ml in 5% dextrose) with saline. All doses of salts are expressed as parent compound equivalent. Gifts of buprenorphine naloxone and tilidine are gratefully acknowledged.

Results

Evaluation of the effects of morphine, buprenorphine and tilidine given subcutaneously

Antinociceptive effects Preliminary experiments indicated that maximal antinociceptive effects occurred 30 min after subcutaneous administration. Morphine, 0.03–10.0 mg/kg, caused dose-dependent significant increases in nociceptive threshold in both hot plate and paw pressure tests (Figure 1a and b). Maximal antinociceptive effects were obtainable at 1.0 mg/kg. In marked contrast, buprenorphine, 0.001–10.0 mg/kg, showed differing profiles of antinociception in the two tests. In the hot plate test a bell-shaped dose-response curve was obtained which reached maximum at a dose of 1.0 mg/kg. The slope of the ascending portion was not as steep as for morphine over the same dose range (Figure 1a). However, in the paw pressure test the shape of the curve was sigmoidal and was linear and steep in the effective submaximal range. Maximal antinociception was achieved and maintained even after a hundred fold increase in the dose (Figure 1b). Tilidine, 5.0–80.0 mg/kg, was only weakly active in the hot plate test (Figure 1a) but was more potent in the pressure test (Figure 1b). Behavioural effects precluded further investigation of higher doses but the portion of the response curve obtained in the pressure test did not differ significantly with respect to slope from those for morphine and buprenorphine. Since the response curves for the three drugs in the hot plate test were not parallel, it was not possible to determine potency ratios. However, comparison of equieffective antinociceptive doses (Table 1) shows that while morphine was slightly more potent in the hot plate test, buprenorphine was 15 times and tilidine 4 times more potent in the paw pressure test than in the hot plate test.

Naloxone (1 mg/kg i.p.) pretreatment alone had no antinociceptive effect but did prevent the antinociceptive effects of all three analgesics in both tests with the exception of tilidine in the paw pressure test (Figure 2).

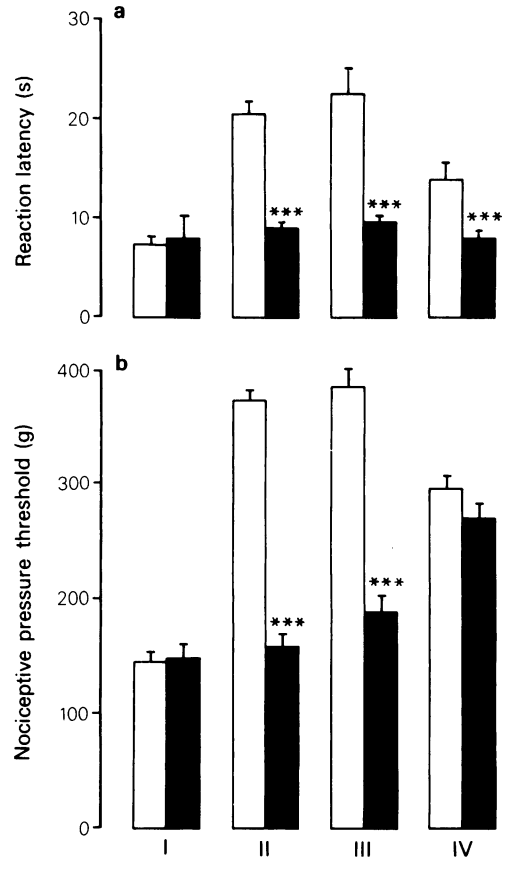

Figure 2 Antinociceptive effects of morphine, buprenorphine and tilidine 30 min after subcutaneous administration to rats pretreated with naloxone. Columns are the means of 7 or 8 animals evaluated in the hot plate test (a) and paw pressure tests (b). Vertical lines indicate s.e.mean. Pretreatments: saline, open columns; naloxone, (1 mg/kg i.p.; 15 min), solid columns. Subcutaneous treatments: (I) vehicle controls; (II) morphine 0.3 mg/kg; (III) buprenorphine, 0.1 mg/kg; (IV) tilidine, 20.0 mg/kg. Statistical analysis of saline v naloxone pretreatment (Student's *t* test): ***$P < 0.001$.

Table 1 Equieffective antinociceptive doses (EAD) of analgesic drugs against thermal and pressure-induced pain in the rat

	Antinociceptive activity (EAD)			
	Subcutaneous (mg/kg)		*Median raphe* (μg/rat)	
Test	*Hot plate*	*Paw pressure*	*Hot plate*	*Paw pressure*
Morphine	0.045	0.13	1.05	0.61
Buprenorphine	0.018*	0.0012	0.017*	0.0034
Tilidine	50.0	12.0	10.5	5.0

EADs are derived from the dose-response curves shown in Figures 1 and 4.
*Values for buprenorphine were calculated from the ascending portion of its dose-response curves.

Overt behavioural changes At higher doses morphine (3.0 and 10.0 mg/kg) and tilidine (80 mg/kg) depressed spontaneous activity, general arousal (as indicated by the decrease in reactivity, touch response, startle response and alertness) and sensoromotor reflex activity. These effects varied in time of onset and duration and were not necessarily associated with the antinociceptive activity of the drugs.

Evaluation of effects following administration of morphine, buprenorphine and tilidine to the median raphe nucleus

Histological verification of injection sites Post mortem histological examination showed that 90% of the cannulae were accurately placed with tips lying within MR. Survey of 20 correctly and 20 incorrectly positioned cannulae demonstrated that significant antinociceptive effects were only observed after micro-injection of analgesic through accurately located cannulae (Figure 3). Data from animals with incorrectly positioned cannulae have been excluded from the following results.

Antinociceptive effects Peak antinociceptive activity of the analgesics in the hot plate and paw pressure tests occurred 10 min after their administration into the MR and are presented in Figure 4. Morphine 1–10 μg/rat, and tilidine 5–80 μg/rat, demonstrated dose-dependent antinociceptive activity in the hot plate test whilst buprenorphine 0.001–1.0 μg/rat, induced its typical bell-shaped dose-response curve with a maximum at 0.1 μg/rat (Figure 4a). There was a linear correlation between log dose and response for the ascending dose-response curves and the slopes did not differ significantly from parallelism. Determination of antinociceptive activity using the paw pressure method showed dose-dependent effects for all three analgesics (Figure 4b). The antinociceptive activity of buprenorphine, 0.001–0.03 μg/rat, was dose-dependent achieving its

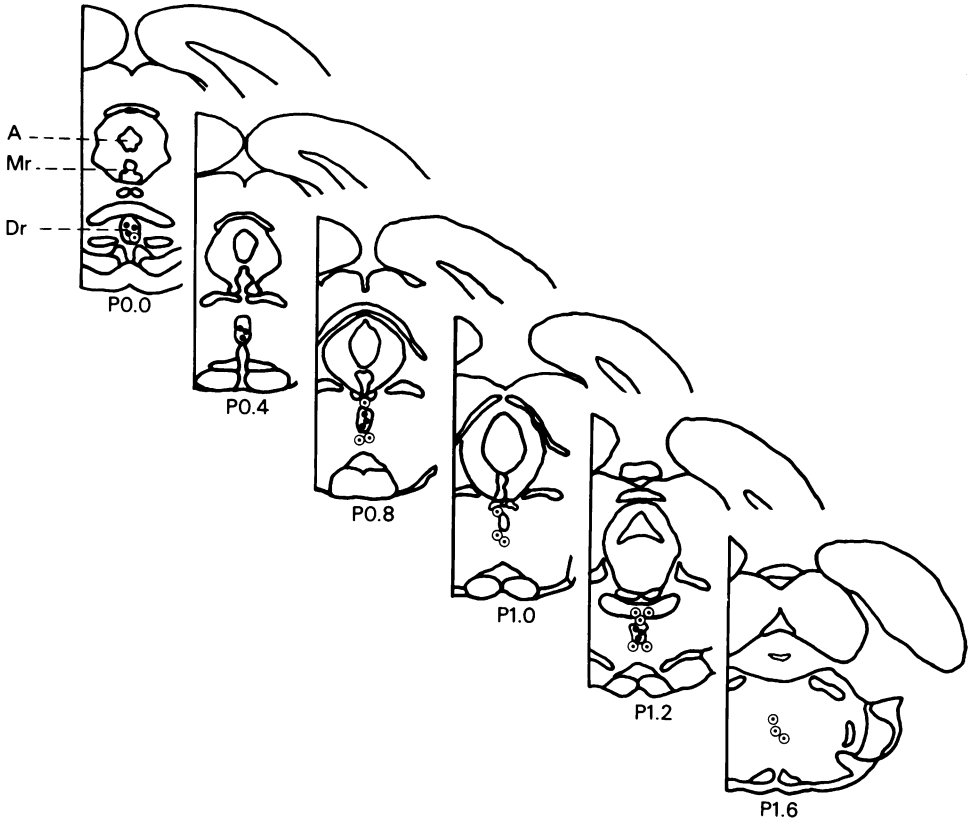

Figure 3 Diagrammatic representation of the localization of injection sites within the medial raphe nucleus of the rat, at which morphine, buprenorphine or tilidine were found (●) or were not found (⊙) to produce antinociceptive effects. Diagrams were constructed from the histological data obtained from 20 rats with the aid of the stereotaxic atlas of Pellegrino & Cushman (1967). MR = medial raphe; DR = dorsal raphe; A = aqueduct.

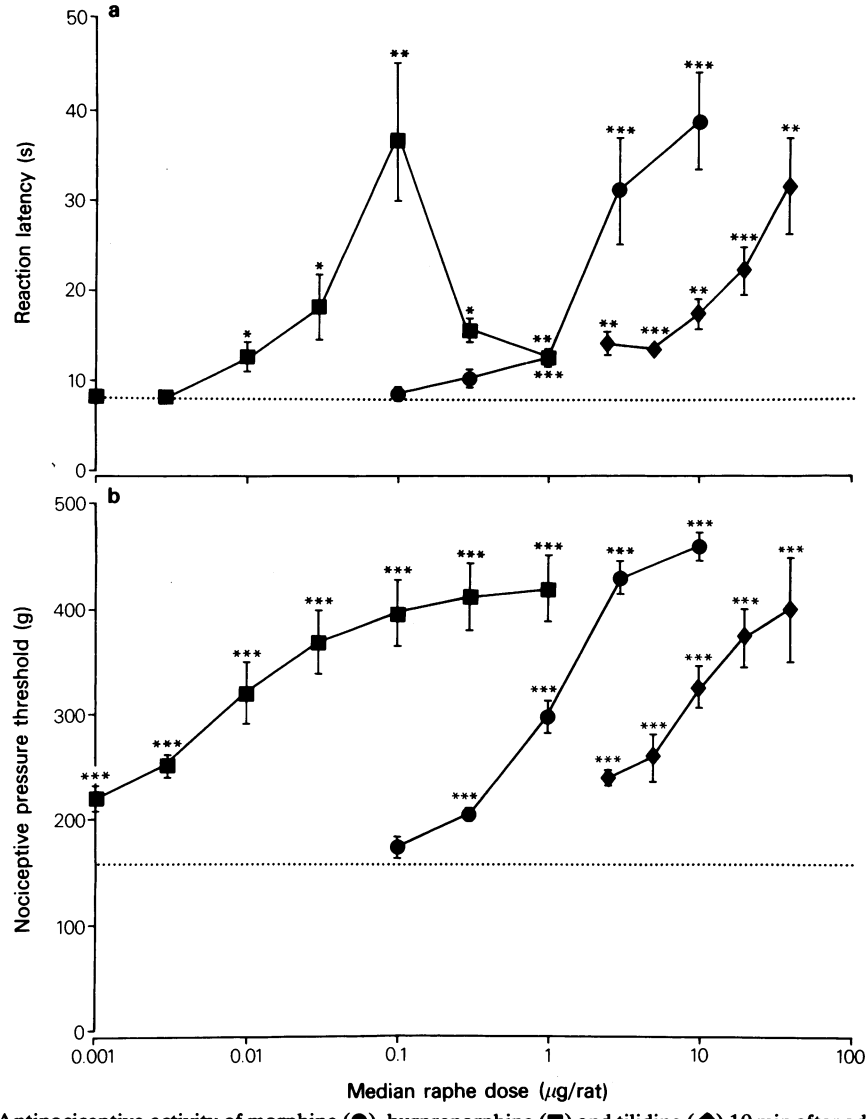

Figure 4 Antinociceptive activity of morphine (●), burprenorphine (■) and tilidine (◆) 10 min after administration to the median raphe in the conscious rat, evaluated in the hot plate test (a) and paw pressure test (b). Each point represents the mean post-drug nociceptive threshold from 5 rats. Vertical lines indicate s.e.mean. Broken lines indicate pre-drug mean nociceptive thresholds. Statistical comparison of pre- and post-drug thresholds (Student's *t* test): $*P<0.05$; $**P<0.01$; $***P<0.001$.

maximum effect at 0.03 μg/rat and this was maintained up to 1.0 μg/rat. The slope of the dose-response curve was shallower than those for morphine, 0.3–10.0 μg/rat, and tilidine, 2.5–40 μg/rat, which were parallel. The maximum antinociceptive effect, limited by the cut-off point in each test, was not observed with any drug treatment. Higher doses of the analgesics were not tested since effects on behaviour were evident and could have impaired the animals' ability to respond to stimuli.

Micro-injection of saline into the MR did not modify nociceptive thresholds at any time. The onset of antinociceptive effects for all three analgesics in both tests was 5 min; however, their duration of action differed. Morphine lasted from 40–90 min, whereas buprenorphine and tilidine lasted for only 20 min. Equi-effective antinociceptive doses for morphine, buprenorphine and tilidine at peak activity after micro-injection into the MR are shown in Table 2.

The effects of naloxone pretreatment (1 mg/kg i.p., 15 min) on the antinociceptive activities of the analgesics are presented in Figure 5. Naloxone pretreatment prevented all antinociceptive activity with the marked exception of that of tilidine in the paw pressure test when no significant antagonism was observed. Naloxone treatment alone did not modify nociceptive thresholds.

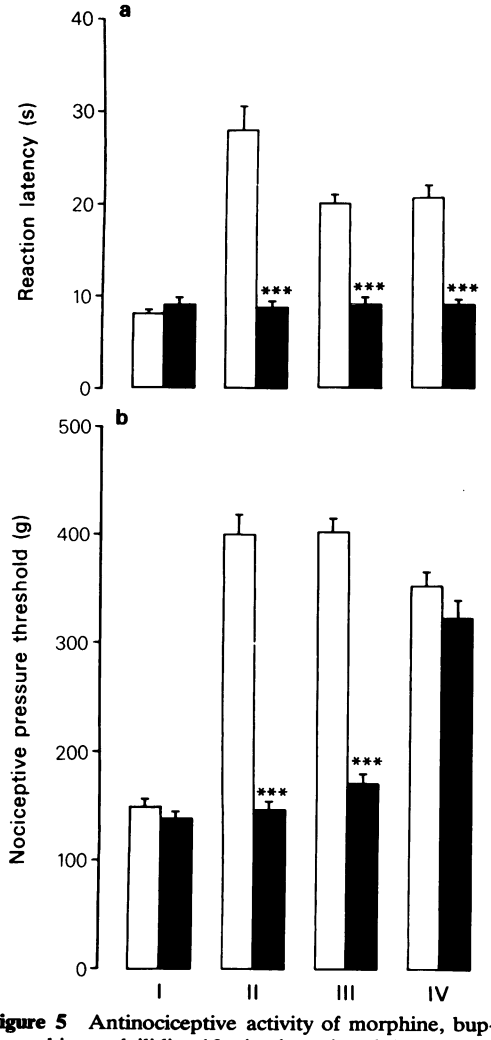

Figure 5 Antinociceptive activity of morphine, buprenorphine and tilidine 10 min after micro-injection into the median raphe nucleus of rats pretreated with naloxone. Columns are the means of 5 or 6 animals evaluated in the hot plate test (a) and the paw pressure test (b). Vertical lines indicate s.e.mean. Pretreatments: saline, open columns; naloxone (1 mg/kg i.p., 15 min) solid columns; MR micro-injection: (I) vehicle control; (II) morphine, 3.0 μg/rat; (II) buprenorphine, 0.1 μg/rat; (IV) tilidine, 20 μg/rat. Statistical analysis of saline v naloxone pretreatment (Student's *t* test: $***P < 0.001$.

Overt behavioural changes Stereotypic behaviour such as biting and gnawing was observable briefly during the 2 min micro-injection procedure. The effects were also induced by saline suggesting that they were injection artifacts and not drug effects. Morphine 3 and 10 μg/rat, caused slight to moderate behavioural depression, Straub tail and increase in limb tone which became more marked with increasing dose and persisted for 55 min. Catalepsy developed 15 min after morphine, 10 μg/rat, but was not consistently evident when antinociception was maximal. The dose-behavioural response relationship for buprenorphine appeared bell-shaped, maximal effects occurring after 0.1 μg/rat. The main effects of buprenorphine were decreases in spontaneous activity and respiratory rate together with increases in body tone. These developed by 15 min and lasted throughout the period. Tilidine had little effect at 7 min except depression of spontaneous activity and respiratory rate and increased limb and body tone. However, by 15 min at doses of 20 and 40 μg/rat, an intense fine tremor developed, there were slight decreases in spontaneous activity, reactivity, touch, altertness, flexor response and respiratory rate with increases in body tone and abnormal gait which persisted for the duration of the session. In general, although seen frequently, the behavioural effects observed following MR administration were only slight within the antinociceptive dose ranges and differed in time course. Therefore, it is unlikely that the raised nociceptive thresholds could have been due to general behavioural disturbance.

Discussion

Following subcutaneous administration of morphine, buprenorphine and tilidine, reliable reproducible antinociceptive activity without significant sensory or motor deficit was demonstrated in both hot plate and paw pressure tests. The profiles of activity, with respect to shape and location of the dose-response curves, correlate well with those reported by Tyers (1980), Cowan, Lewis & MacFarlane (1977) and Hermann *et al.* (1970). The ability of naloxone to prevent the development of these antinociceptive properties, with the notable exception of tilidine in the pressure test, supports current concepts of the involvement of opiate receptors. Since the postulated activity profiles of the analgesics were confirmed after subcutaneous administration their effects after micro-injection into the median raphe were evaluated.

Administration directly into the median raphe by micro-injection in the conscious rat showed, firstly, that all three analgesics possess antinociceptive activity and induce some behavioural changes. However,

for all three drugs, there was clear discrimination with respect to time, frequency of occurrence and intensity of effect between antinociception and observed behavioural effects such as catalepsy, motor impairment and non-specific sensoro-motor deficit which may falsely indicate antinociceptive activity. At the highest doses tested there was more general impairment of function so that the specificity of antinociception is more doubtful at these doses. Maximal antinociceptive activity, limited only by the cut off conditions of the test, was not demonstrated for any of the analgesics. Secondly, there was complete correlation of the site of drug delivery with the presence or absence of antinociceptive activity. This, together with the rapid onset of activity before most other observable effects supports the hypothesis that the MR was indeed the anatomical substrate involved in the antinociceptive activity of the drugs. An injection artefact was detectable but was short-lived and clearly differentiable from drug-induced effects. It was not possible from these experiments to assess whether the behavioural effects were induced locally or more remotely by diffusion, therefore, it is valid to comment on whether these effects influenced the apparent antinociceptive activity of the drugs but it is not valid to make inferences about the role of the median raphe in the behavioural effects observed. Qualitatively similar antinociceptive activity was seen after both subcutaneous and MR administration (see below). In particular, the bell-shaped dose-response curve for buprenorphine obtained after peripheral injection was replicated when injection was made into the MR.

Quantitative differences are apparent following administration by peripheral and central routes, but lack of parallelism of the ascending portion of the dose-response curves precludes precise numerical quantitation of relative potencies of the analgesics. Consideration of effective analgesic doses indicates approximately equivalent effects with doses in μg/rat MR and mg/kg SC with the exceptional lack of potency (10–100 fold) of morphine MR, particularly in the hot plate test. These data and those of Yaksh & Rudy (1976) are not irreconcilable. The single dose of morphine (5 μg/rat) used by Yaksh as shown in our laboratory would have been only just above the threshold of effect. The relative lack of potency of morphine MR, particularly in the hot plate test, also indicates that other sites may be more important for this facet of antinociception. Another factor could be the differences between the analgesics with respect to metabolism and passage through the blood brain barrier. The activity of buprenorphine MR, is consistent with data from Rance & Shillingford (1976) who demonstrated extensive peripheral metabolism of the compound, but correlated antinociceptive effect with the presence of the parent drug rather than the metabolite in the brain.

Tilidine activity is more controversial. Tilidine is extensively metabolized, peripherally, to two active metabolites, nortilidine (I) and bis-nortilidine (II) but only tilidine and nortilidine pass the blood brain barrier (Schulz, Blasig, Wuster & Herz, 1978). Further Dubinsky, Crew, Melgard, Karpowicz & Di Carlo (1975) correlated plasma and brain concentration of nortilidine but not tilidine with antinociception in the tail flick test. In the absence of other data it would seem that the parent compound does have appreciable antinociceptive activity when microinjected directly into MR but activity following peripheral administration may result from the parent and/or its metabolite, nortilidine, which may possess a similar profile of activity. More extensive experiments would be necessary to determine whether the naloxone-insensitive activity of tilidine is truly non-opioid but the present results indicate that tilidine acting in the MR can participate in pressure antinociception without the involvement of opiate receptors.

The demonstration of SPA (Reynolds, 1969) initiated speculation and research into mechanisms which can modulate the perception of pain (Mayer & Price 1976). One system is that proposed by Fields & Basbaum (1978) who provide evidence for a descending inhibitory system from the medullary NRM to the cord involving 5-HT fibres in the dorsolateral funiculus. Oleson *et al.* (1978) proposed the inclusion of the mid- and hind-brain raphe nuclei in a descending 5-HT inhibitory system; however, the paucity of caudal projections from the MR in the rat (Conrad, Leonard & Pfaff, 1974) would not support this hypothesis although the MR may contribute indirectly by means of its interconnections with the dorsal raphe nucleus (DR) (Mosko, Haubrich & Jacobs, 1977).

The most marked feature of the MR and DR nuclei is that the cell bodies which provide the 5-HT fibres for the ascending projection from the reticular system to the forebrain are located here (Dahlström & Fuxe, 1964; Fuxe & Jonsson 1974; Bowsher, 1976). Dissociation to some extent of the roles of the DR and MR in antinociception is possibly indicated by their differing sensitivities to the effects of morphine microinjection (Yaksh & Rudy, 1976) and by the effects of specific lesions (Adler *et al.*, 1975). Azmitia & Segal (1978) and Bobillier, Petitjean, Salvert, Ligier & Seguin (1975) have demonstrated overlapping but differential projections of these extensive rostral 5-HT systems in the rat and cat respectively. The MR receives afferents from the B9 area (Dahlström & Fuxe, 1964; nomenclature) and the medial aspect of the lateral habenular nucleus (Aghajanian & Wang, 1977; Herkenham & Nauta, 1979), which in turn receives most of its input from

limbic regions. The afferent and efferent anatomical connections of the MR therefore, although not precluding a role for it in drug-induced antinociception involving a descending inhibitory system, are more consistent with a hypothesis involving the ascending 5-HT projection from the reticular formation to limbic areas. Altered median raphe activity may then be more likely to influence the perception and emotional qualities of painful stimulation.

Summarizing, we find that opioid events are associated with antinociceptive microinjection of analgesics into the MR. These three analgesics evoked the full spectrum of their activities in the MR, which includes μ- and κ-opiate receptor activation (Tyers, 1980) in addition to the non-opioid activity of tilidine in the pressure test. The indications are, however, that the MR is not the only site involved in drug-induced antinociception and that there is more relevance to pressure than thermal-induced nociception.

The MR nucleus might be involved in ascending and/or descending pathways associated with pain perception. The possibility of drug-induced regulation of function in forebrain 5-HT systems should be considered.

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