Opioid inhibition of rat periaqueductal grey neurones with identified projections to rostral ventromedial medulla *in vitro*

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1. Rat caudal periaqueductal grey (PAG) output neurones containing rhodamine microspheres, retrogradely transported from an injection site in the rostral ventromedial medulla (RVM), were visualized in brain slices and recorded from using whole-cell patch clamp techniques.

2. The specific $GABA_B$ receptor agonist baclofen (10 μM) produced an outward current or hyperpolarization in fifty out of fifty-six caudal PAG output neurones. In 44% of these baclofen-sensitive neurones, the opioid agonist methionine enkephalin (30 μM) also produced an outward current or hyperpolarization. The opioid current reversed polarity at −104 mV and could also be produced by DAMGO, an agonist selective for the μ-subtype of opioid receptor.

3. Opioid-responding output neurones were not distributed uniformly in the caudal PAG. In horizontal slices containing lateral PAG, 56% of output neurones were inhibited by opioids, as compared with only 14% of the output neurones in slices containing ventrolateral PAG.

4. These observations are consistent with opioid disinhibition of ventrolateral PAG neurones projecting to the RVM as the predominant mechanism underlying opioid-induced analgesia in the PAG. The role of opioid receptors found on a major proportion of the output neurones in the lateral PAG remains to be established, but is assumed not to be related to modulation of nociceptive function.

The periaqueductal grey (PAG) is a neurone-dense region that surrounds the midbrain aqueduct. There is evidence that two separate, and distinct, nociceptive modulatory systems operate in the caudal PAG: a dorsal system which encompasses the dorsomedial, dorsolateral and lateral subdivisions of the PAG, and a ventral system which includes the ventrolateral PAG and also the dorsal raphe (reviewed by Morgan, 1991). Opioids appear to interact exclusively with the ventral system, as antinociception produced by electrical stimulation of ventral, but not dorsal, PAG regions is attenuated by the opioid antagonist naloxone (Cannon, Prieto, Lee & Liebeskind, 1982). Furthermore, microinjection of low concentrations of the opioid agonist morphine produced antinociception only when microinjected into the ventrolateral PAG region (Yaksh, Yeung & Rudy, 1976).

Both PAG nociceptive modulatory systems act via descending pathways that include a relay in the rostroventral medial medulla (RVM) – a region which encompasses the nucleus raphe magnus and the adjacent paramedian reticular formation. In rat, as many as 18% of PAG neurones project to the RVM. These cells are distributed throughout the dorsomedial, lateral and ventrolateral PAG divisions, but are absent in the dorsolateral PAG division as well as in and around the dorsal raphe nucleus (Reichling & Basbaum, 1990).

To explain opioid-induced analgesia in the PAG, neural circuits have been postulated that are based on the assumptions that: (1) opioids only act *directly* to inhibit PAG neurones; and (2) that excitatory opioid effects are caused *indirectly*, i.e. via disinhibition (see Morgan, Heinricher & Fields, 1992). The first of these assumptions is well supported by intracellular microelectrode studies in rat brain slices which have shown that opioids act via μ-receptors to directly hyperpolarize subpopulations of neurones predominantly located in the lateral PAG (Behbehani, Jiang & Chandler, 1990; Chieng & Christie, 1994*a*). The second assumption, opioid disinhibition, was first proposed to reconcile the seemingly conflicting observation that opioids (assumed to be inhibitory) have the same effect as PAG stimulation in causing antinociception. Although much indirect evidence supports this model (Reichling, 1991), no experiments have directly demonstrated opioid-induced disinhibition of PAG neurones

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which project to the RVM. Experiments in brain slices have found that opioids produce roughly equal inhibition of both glutamatergic and GABAergic electrically evoked synaptic potentials (Chieng & Christie, 1994*b*) recorded in PAG neurones. These results suggested that opioids might act in PAG by mechanisms other than simple disinhibition of descending projection neurones involved in antinociception.

In the present study we have directly examined the opioid sensitivity of neurones in lateral and ventrolateral PAG which project to the RVM. Following injection of rhodamine-conjugated microspheres into the RVM of rats, brain slice preparations were used to make whole-cell patch clamp recordings from visualized neurones (Nomarski and fluorescence optics) that contained retrogradely transported label.

METHODS

Experiments were performed according to Australian National Health & Medical Research Council guidelines. Injection of rhodamine-conjugated latex microspheres (Lumofluor, New York, USA) into the rostral ventromedial medulla was performed using 8- to 12-day-old Sprague–Dawley rats. The animals were maintained under general anaesthesia (1–2% halothane in 100% O_2), placed in a stereotaxic frame and the dura exposed by trephination. Using glass micropipettes, four to five separate 30 nl injections were made so as to achieve a final volume of 120–150 nl. Injection volumes were measured using a calibrated reticule installed in an operating microscope. Following surgery, rats were allowed to recover from anaesthesia in a warmed box before being returned to holding boxes with their mother and litter.

Between 2 and 7 days post-surgery, rats were anaesthetized (halothane), decapitated, and their brains quickly removed and immersed in ice-cold artificial cerebrospinal fluid (ACSF) containing (mM): NaCl, 126; KCl, 2·5; NaH_2PO_4, 1·4; $MgCl_2$, 1·2; $CaCl_2$, 2·4; glucose, 11; $NaHCO_3$, 25; and equilibrated with 95%O_2 : 5% CO_2. A vibratome was used to prepare six to eight horizontal slices (200–250 μm thickness) containing the midbrain periaqueductal grey, which were placed in a holding chamber containing oxygenated ACSF maintained at 32 °C.

Injection sites were examined for accuracy in either coarse dissected fresh tissue, or in medulla that was blocked separately, fixed in phosphate-buffered (0·1 M, pH 7·2) 4% paraformaldehyde and subsequently sectioned. In fixed tissue, injection tracks were located in 100 μm vibratome sections of medulla only if successful recordings of retrogradely labelled PAG neurones had been made, i.e. only from midbrains containing large numbers of rhodamine-bead labelled PAG neurones. The locations of injection sites were plotted with reference to alternating counterstained sections (Neutral Red), and to the atlas of Paxinos & Watson (1986).

In preparation for electrophysiological recording, brain slices were placed in a chamber (1·5 ml volume) mounted on the stage of an upright microscope (Olympus BH-2 with a fixed-stage modification) and viewed using a water-immersion objective (Zeiss, ×40). Slices were continuously superfused (2 ml min^{-1}) with ACSF (32 °C). Neurones located in the caudal PAG that contained rhodamine label were identified under fluorescent epi-illumination and could be easily distinguished without additional image intensification. Once a labelled neurone (referred to as PAG–RVM neurones) had been identified, it was viewed using Nomarski optics and patch clamp recordings were made using standard techniques (Edwards, Konnerth, Sakmann & Takahashi, 1989). In brief, neurones were 'cleaned' using a micropipette containing ACSF and then whole-cell recordings were made using patch electrodes (4–6 MΩ) containing (mM): potassium gluconate, 125; NaCl, 15; $MgCl_2$, 2; Hepes, 10; EGTA, 11; MgATP, 2; NaGTP, 0·25; biocytin or neurobiotin, 0·3%; adjusted to a pH of 7·3 with KOH and an osmolarity of 270–290 mosmol l^{-1}. Liquid junction potentials of −11 mV were calculated using JPCalc (Barry, 1994) and corrected for during subsequent data analysis. At completion of recording the location of the neurone was mapped.

Following some experiments, slices were fixed in phosphate-buffered 4% paraformaldehyde. Biocytin (Sigma) or neurobiotin (Vector, Burlingame, CA, USA) filled neurones were visualized after staining with FITC-conjugated avidin (Amersham) as previously described (Chieng & Christie, 1994*a*).

Stock solutions of all drugs were made using distilled water. These were diluted to working concentrations using ACSF and applied by superfusion. Baclofen and methionine enkephalin (met-enkephalin) were obtained from Sigma. DAMGO ([D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]-enkephalin) was generously supplied by the National Institute on Drug Abuse (Bethesda, MD, USA).

RESULTS

Detailed examination was made of nineteen injection sites from which thirty-two recordings were made from labelled PAG neurones. In every case, injections of rhodamine beads were restricted to within 1 mm of the mid-line, directly dorsal to the pyramids (Fig. 1). Rostrocaudally, all of the series of injections occupied a volume of ventromedial medulla bounded by the caudal limit of the trapezoid body and the rostral olivary complex (approximately 10·3 to 12·3 mm caudal to bregma in adult rats as estimated from the atlas of Paxinos & Watson, 1986). The majority of injections ($n = 9$) were made directly into the nucleus raphe magnus, extending from 10·3 to 11·3 mm caudal, and from 9 to 11 mm ventral to bregma. Fewer labelled neurones were found in PAG slices when injections did not involve the nucleus raphe magnus. No relationship was found between the location of injection sites and the nature of the agonist response measured in labelled PAG neurones (see below; Fig. 1).

Patch clamp recordings were made from fifty-six PAG–RVM neurones that contained fluorescent rhodamine microspheres. Although cells containing fluorescent microspheres could be visually identified and reliably targeted with recording electrodes, some neurones were also filled with biocytin or neurobiotin for *post hoc* confirmation with FITC-avidin staining (see Fig. 1). This experiment was attempted after seventeen recordings, of which seven neurones were successfully stained. We attribute the low number of successfully stained neurones to damage caused when detaching the large, low-resistance recording pipettes. In all cases where FITC-labelled cells were clearly identified,

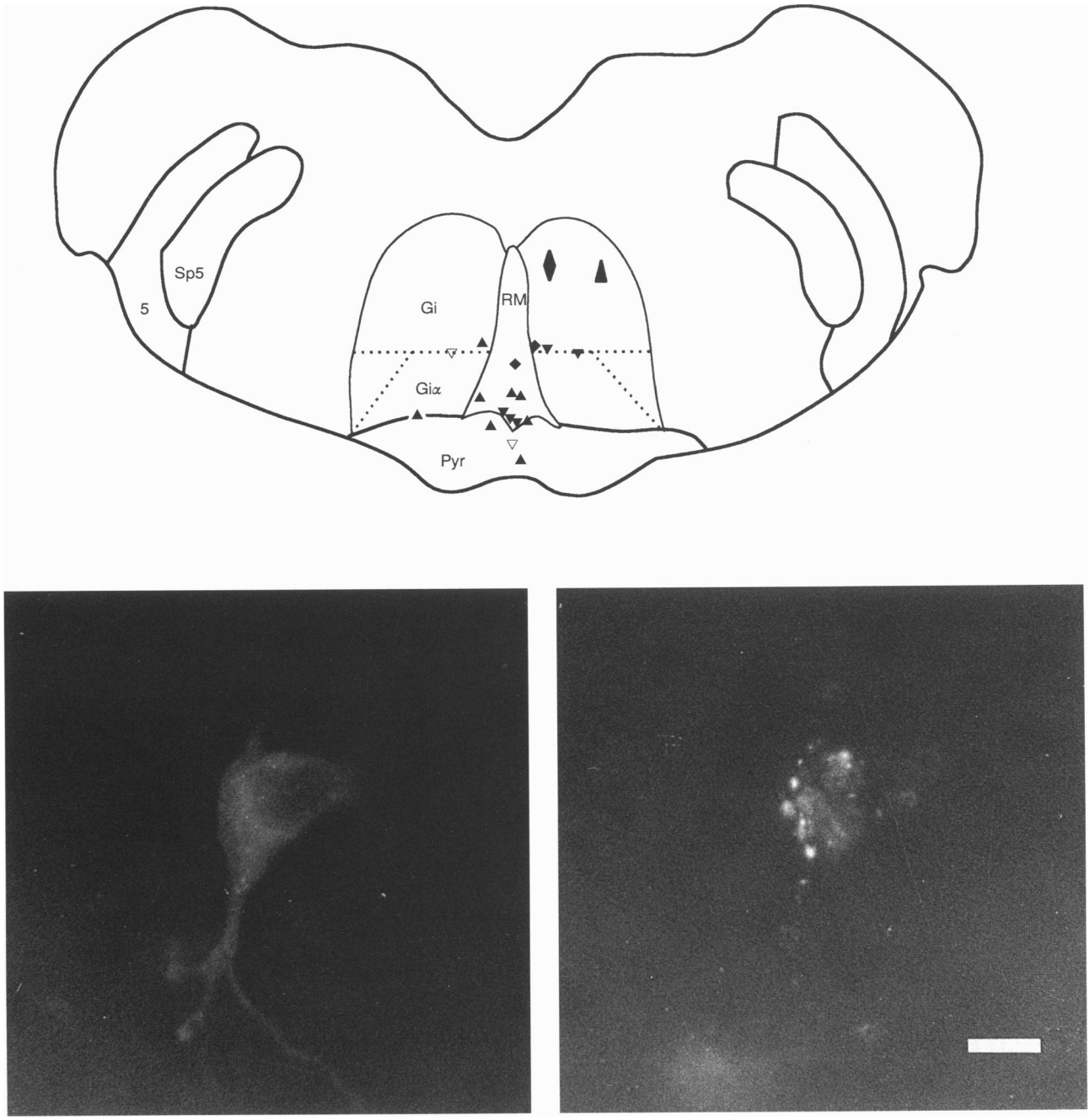

Figure 1. Visualization of injection sites and PAG–RVM projection neurones

The upper panel shows the ventral limits of 19 injections of rhodamine microspheres (made from the dorsal surface) into the rostromedial medulla. The diagram was prepared from a stereotaxic atlas of the rat brain (Paxinos & Watson, 1986) and represents a transverse section made at 12·6 mm caudal to bregma in an adult rat. From these injections, a total of 32 recordings were made from PAG–RVM output neurones. Indicated are sites that labelled opioid-responding (▲), opioid-insensitive (▼), or both classes (◆) of neurones in PAG. Rostrocaudally, the injection sites were centred in a volume bounded by the caudal edge of the trapezoid body (10·3 mm caudal to bregma in adult rats) and the rostral limits of the olivary complex (11·8 mm caudal to bregma), except for 2 sites (▽) which were level with the rostral olivary complex. Abreviations: 5, 5th spinal nerve; Gi, paragigantocellular reticular nucleus; Giα, paragigantocellular pars alpha; Pyr, pyramids; RM, nucleus raphe magnus; Sp5, nucleus of the 5th nerve. In the lower panel, the left photomicrograph represents a biocytin-filled PAG neurone visualized using FITC-conjugated avidin. In the same view in the right micrograph, co-localized rhodamine microspheres can be visualized in the filled neurone, establishing it as a PAG–RVM projection neurone. Scale bar, 10 μm.

the neurones were also found to contain rhodamine fluorescent microspheres. Even with this restricted sample ($n = 7$), neurones were characterized with morphologies typical of each of three major classes of PAG neurones: fusiform ($n = 2$), triangular ($n = 3$) and multipolar ($n = 2$) (Chieng & Christie, 1994*a*). The mean soma diameter (measured along the longest axis) was 19 ± 3 μm. Soma shape or diameter did not clearly differ between opioid-sensitive and -insensitive neurones (see below).

In the majority of experiments, PAG–RVM neurones were voltage clamped to a potential of −60 mV, although some neurones were studied using current clamp. Superfusion with the $GABA_B$ receptor agonist baclofen (10 μM) produced an outward current, or a hyperpolarization (in one case an outward current was produced by noradrenaline, 30 μM, rather than baclofen), in fifty of fifty-six labelled output neurones from which stable recordings were obtained (see Fig. 2). Of these cells, 44% (22 of 50) also produced an outward current or hyperpolarization when superfused with met-enkephalin (30 μM, see Fig. 2). The mean amplitude of the agonist currents in the output neurones was 37 ± 8 pA for met-enkephalin ($n = 21$) and 38 ± 4 pA for baclofen ($n = 43$). In opioid-responding neurones, the baclofen current was 40 ± 8 pA in amplitude ($n = 19$), and in opioid-insensitive neurones it was 34 ± 4 pA ($n = 24$). The six output neurones which responded to neither baclofen nor met-enkephalin in this population were not considered further because baclofen hyperpolarized all PAG neurones

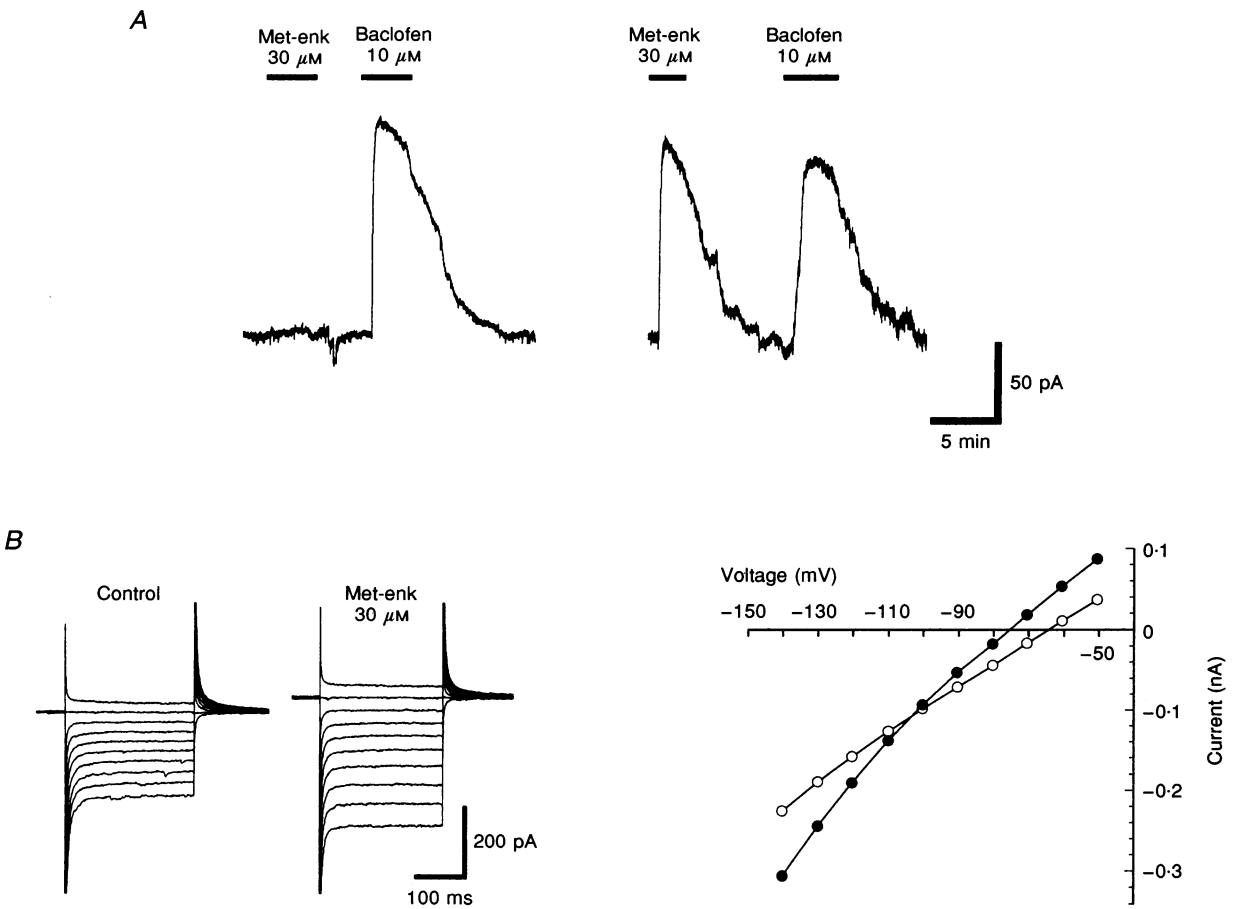

Figure 2. Agonist currents in PAG–RVM neurones

Membrane currents measured in caudal PAG–RVM neurones. Neurones were clamped to a command potential of −60 mV. *A*, in the left panel is a recording from a projection neurone in which met-enkephalin (met-enk; 30 μM) had no effect but baclofen (10 μM) produced an outward current; in the right panel is a recording from an output neurone where both agonists caused outward currents. *B*, in the left panel, voltage command steps 200 ms in duration were made in 10 mV increments from −50 to −140 mV from a holding potential of −60 mV. Shown are the resulting currents observed in a single PAG–RVM neurone in the absence and presence of met-enkephalin (30 μM). The amplitudes of these evoked currents were measured and are plotted in the right panel. Current–voltage relationships in 0 and 30 μM met-enkephalin are indicated by the symbols ○ and ●, respectively.

tested in a previous study from this laboratory using intracellular electrodes (Chieng & Christie, 1995).

The hyperpolarization produced by met-enkephalin in PAG neurones was previously characterized and attributed to a μ-opioid receptor-mediated increase in an inwardly rectifying potassium conductance (Chieng & Christie, 1994*a*). In the present study, the outward current produced by met-enkephalin (30 μM) was mimicked by the μ-receptor selective agonist, DAMGO (3 μM). Current–voltage relationships were measured in three opioid-sensitive output neurones prior to and during superfusion with met-enkephalin (30 μM). The resting conductance showed inward rectification with slope conductances of $1{\cdot}9 \pm 0{\cdot}4$ nS and $2{\cdot}9 \pm 0{\cdot}3$ nS measured between −60 to −90 mV and −110 to −130 mV, respectively. Met-enkephalin significantly increased the conductances to $2{\cdot}5 \pm 0{\cdot}6$ nS and $4{\cdot}2 \pm 0{\cdot}8$ nS, respectively when measured over the same potentials ($P < 0{\cdot}05$, 2 d.f., Student's paired t test). The opioid-induced current reversed polarity at -103 ± 2 mV ($n = 3$). This is in close agreement with the Nernst equation value of −104 mV predicted from the composition of intracellular and extracellular solutions. An example of measurements made in a single neurone is shown in Fig. 2.

The locations of the PAG–RVM neurones from which recordings were made are shown in Fig. 3. These have been mapped to three horizontal levels which approximate to bregma −5·1, −5·6 and −6·1 mm as defined for adult rats by Paxinos & Watson (1986). The proportion of opioid-sensitive neurones differed significantly between these levels ($\chi^2 = 6{\cdot}97$, 2 d.f., $P < 0{\cdot}05$). Fifty-six per cent (10/18) of neurones within the lateral PAG (i.e. at the most dorsal level, bregma −5·1 mm) were opioid-responding, 56% (10/18) at the lateral–ventrolateral border (bregma −5·6 mm), and only 14% (2/14) of neurones in the ventrolateral PAG (i.e. at the most ventral level, bregma −6·1 mm). The proportion of opioid-responding neurones in slices located near −6·1 mm ventral to bregma differed from the other two levels combined ($\chi^2 = 5{\cdot}39$, 1 d.f., $P < 0{\cdot}05$). The distribution in the two more dorsal sections (−5·1 and −5·6 mm ventral to bregma) did not differ ($\chi^2 = 0{\cdot}11$, 1 d.f., $P > 0{\cdot}7$).

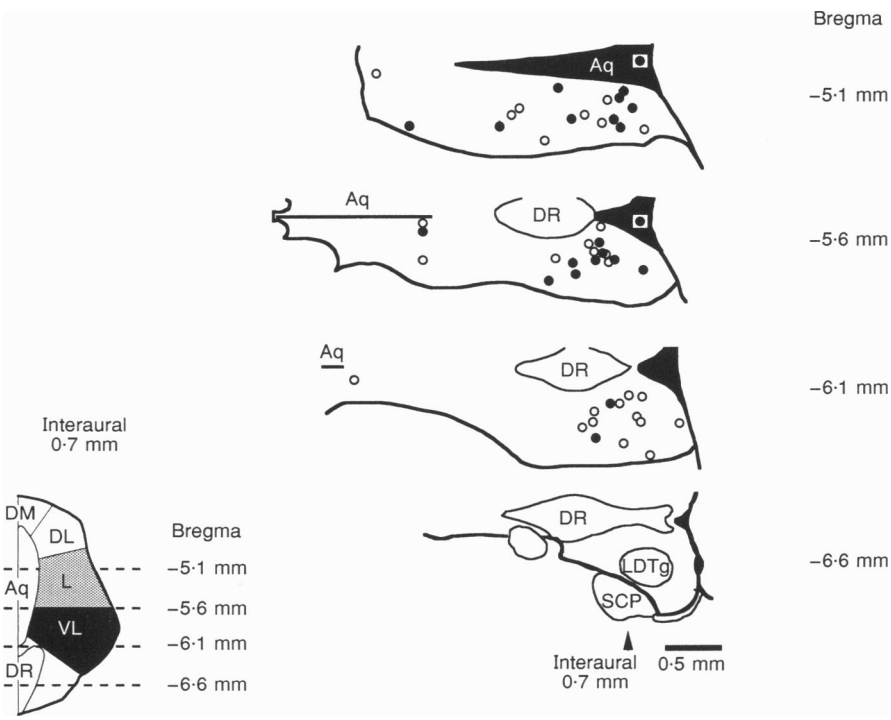

Figure 3. Locations of PAG–RVM neurones

Anatomical locations of opioid-responding (●) and opioid-insensitive (○) PAG–RVM projection neurones in three dorsoventral levels of horizontal PAG sections (approximating to 5·1, 5·6 and 6·1 mm ventral to bregma). In the diagram at the bottom left is indicated the relation of these sections to the dorsomedial (DM), dorsolateral (DL), lateral (L) and ventrolateral (VL) PAG columns (see Bandler & Shipley, 1994) as viewed in the transverse plane (Interaural 0·7 mm). For two neurones (marked within the aqueduct), the level was determined but not the precise location. Diagrams were prepared from a stereotaxic atlas of the adult rat brain (Paxinos & Watson, 1986), therefore the dimensions relative to bregma, although included for illustration, are not to scale for the immature rats that were used. Abreviations: Aq, aqueduct; DR, dorsal raphe; LDTg, lateral dorsal tegmental nucleus; SCP, superior cerebellar peduncle.

DISCUSSION

In this study we have used retrograde tracing in combination with patch clamp techniques to record from identified PAG–RVM neurones in the caudal PAG. This approach has enabled us to overcome a major limitation of using brain slice preparations for electrophysiological studies as it enables recordings to be made from anatomically identified output neurones which make a known projection to another brain region. The use of rhodamine microspheres as the retrogradely transported marker allowed PAG neurones (labelled from the RVM) to be identified and recorded from using standard optics without additional image intensification (see Kangrga & Loewy, 1994).

The opioid agonist met-enkephalin (30 μM) hyperpolarized, or produced an outward current, in 44% of PAG–RVM neurones. Analysis of the current–voltage relationship of the agonist-induced current revealed that it reversed polarity near the potassium equilibrium potential predicted from the compositions of the internal and external solutions ($E_K = -104$ mV), and was associated with a conductance increase. This confirms a previous study of PAG neurones by our laboratory using intracellular recording (Chieng & Christie, 1994*a*) which found that postsynaptic hyperpolarizing responses to met-enkephalin were mediated exclusively by μ-opioid receptors coupled to an increased potassium conductance. The distribution of opioid-sensitive PAG–RVM neurones was not uniform over the dorsoventral extent of the PAG used for recording, with a significantly smaller proportion of PAG–RVM neurones being directly inhibited by opioids in the ventrolateral PAG. This distribution is similar to that found when recording from the entire PAG neuronal population in our previous study using intracellular electrodes.

Current models of PAG organization subdivide the nucleus into functionally specialized longitudinal columns that run in a rostrocaudal direction (Depaulis, Keay & Bandler, 1992; Cameron, Khan, Westlund, Cliffer & Willis, 1995; see also Bandler & Shipley, 1994). In the present study our recordings were made predominantly in the caudal PAG. Within this region in rat, stimulation of the lateral column characteristically produces flight/forward avoidance behaviour mixed with episodes of freezing, analgesia, and increases in arterial pressure and heart rate. In contrast, stimulation of ventrolateral PAG produces quiescence, hyporeactivity, analgesia and decreases in arterial pressure and heart rate (Bandler & Shipley, 1994). We found that a much smaller proportion of PAG–RVM neurones in the ventrolateral column were directly inhibited by opioids (14%), compared with inhibition by opioids of more than half of the equivalent neuronal population in the lateral PAG region (56%). This pattern is assumed to reflect the functional specialization of the two regions. These functional differences could be related to distinct targets in RVM that are supplied by these subdivisions of PAG.

The effects of opioids injected into the caudal ventrolateral PAG with respect to antinociception have been well characterized. Opioids microinjected into the ventrolateral PAG inhibit nociceptive reflexes such as the tail-flick response to noxious heat. It has also been reported that local application of morphine in the PAG at doses that produce antinociception also suppresses firing of 'on-cells' in the RVM. In RVM, on-cells have been defined *in vivo* by their display of a burst of firing prior to the tail flick produced in response to noxious heat, and have been suggested to have a permissive or facilitating influence upon nociceptive processing (Fields, Barbaro & Heinricher, 1988). Studies of this pathway have proposed that opioid excitation of on-cells is due to an opioid-mediated reduction of tonic inhibitory input to the PAG–RVM neurones (i.e. disinhibition). In the present study, the large proportion (86%) of PAG–RVM projection neurones arising from ventrolateral PAG which were not directly inhibited by opioids is consistent with such a mechanism. However, it is not yet clear whether these neurones would be disinhibited by opioids *in vivo*, as this would require demonstration of selective inhibition of GABAergic tone. In a previous study of PAG neurones which were not directly inhibited by opioids using intracellular electrodes, we observed similar inhibition of both glutamatergic and GABAergic evoked synaptic potentials (Chieng & Christie, 1994*b*). The small proportion of PAG–RVM neurones in ventrolateral PAG that are directly inhibited by opioids could have a role in functions other than antinociception, or alternatively might provide excitatory inputs to on-cells (discussed by Morgan *et al.* 1992).

Opioids inhibit a large proportion of PAG–RVM neurones in the lateral PAG. In both rat and cat, the caudal lateral PAG co-ordinates a group of physiological reactions that relate to a specific form of 'defence' behaviour. Thus, the responses evoked by excitatory amino acids injected into caudal lateral PAG include flight, freezing, antinociception, and increases in blood pressure associated with visceral vasoconstriction and skeletal muscle vasodilatation (Depaulis *et al.* 1992; Lovick, 1993; Bandler & Shipley, 1994). Opioid-sensitive PAG–RVM neurones in lateral PAG are unlikely to be involved in antinociception because opioid microinjections into the lateral PAG have little antinociceptive action (Yaksh *et al.* 1976*b*). Similarly, opioid antagonists have no significant effects on the antinociception produced by stimulation of this area (Cannon *et al.* 1982). Furthermore, the neurones that mediate antinociception from lateral PAG might not project directly to the RVM (Morgan, 1991). The major influence of opioids on lateral PAG–RVM neurones might be on the cardiovascular and/or other non-analgesic functions mediated by these projections.

In conclusion, this study has demonstrated the power of retrograde labelling and patch clamp techniques when used

in combination to record from an identified class of output neurone. This approach is especially valuable when applied to brain regions such as the PAG, the neurones of which are heterogeneous and do not display electrophysiological properties that correspond to their distinct morphological types (Chieng & Christie, 1994*a*). The relatively low numbers of ventrolateral PAG output neurones that are physiologically inhibited by opioids may be consistent with models that propose opioid disinhibition as the predominant mechanism underlying opioid-induced analgesia in the PAG. The significance of the surprisingly high proportion of lateral PAG output neurones that possess functional opioid receptors remains to be determined.

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