Hyperpolarization by opioids acting on μ -receptors of a sub-population of rat periaqueductal gray neurones *in vitro*

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1 The actions of opioids on membrane properties of rat periaqueductal gray neurones were investigated using intracellular recordings from single neurones in brain slices. Morphological properties and anatomical location of each impaled neurone were characterized by use of intracellular staining with biocytin. The present paper primarily considers neurones which were directly hyperpolarized by opioids. The accompanying paper considers inhibition of synaptic transmission by opioids.

2 Met-enkephalin (10–30 μ M) hyperpolarized 29% (38/130) of neurones. The hyperpolarization was fully antagonised by naloxone (1 μ M, $n = 3$). The response to Met-enkephalin was not affected by agents which block synaptic neurotransmission (1 μ M tetrodotoxin, and 0.1 μ M tetrodotoxin + 4 mM Co^{2+} , $n = 3$).

3 The specific μ -receptor agonist, D-ala-met-enkephalin-glyol (3 μ M, $n = 17$) produced hyperpolarizations of similar amplitude to those produced by Met-enkephalin (10–30 μ M). The EC_{50} of D-ala-met-enkephalin-glyol was 80 nM and the maximum response was achieved at 1–3 μ M. The δ -receptor (D-Pen-D-Pen-enkephalin, 3 μ M, $n = 7$) and κ -receptor (U50488H, 3 μ M, $n = 5$) agonists had no effect on the membrane properties of these neurones.

4 The opioid-induced hyperpolarization was associated with an increased potassium conductance. Hyperpolarizations were accompanied by a significant decrease in membrane resistance between –70 and –80 mV, and a significantly greater decrease between –110 and –140 mV ($n = 16$). Hyperpolarizations reversed polarity at -111 ± 3 mV ($n = 16$), close to the expected equilibrium potential for potassium ions. The reversal potential of outward currents increased by 24 mV when the extracellular potassium concentration was raised from 2.5 to 6.5 mM, which is close to the value predicted by the Nernst equation (25 mV) for a potassium conductance.

5 Resting inward rectification (reduced input resistance at potentials more negative than –100 mV in the absence of opioids) was significantly greater in neurones which were hyperpolarized by opioids than in those which were not hyperpolarized. The amplitude of action potential after hyperpolarizations was significantly smaller in neurones which were hyperpolarized by opioids. Other membrane properties did not differ significantly between opioid-sensitive and -insensitive neurones.

6 Neurones hyperpolarized by opioids were multipolar (58%), triangular (21%) or fusiform (5%) in shape with a soma diameter of 22 ± 1 μ m ($n = 19$, longest axis). Dendritic spread was in a large radiating pattern, usually in all directions, with axons usually originating from primary dendrites. The axons were usually branched and projected in several directions. Morphological properties did not differ significantly between opioid-sensitive and -insensitive neurones.

7 Neurones hyperpolarized by opioids were located predominantly in the lateral periaqueductal gray, as well as in the more dorsal areas of the ventrolateral periaqueductal gray, whereas neurones not hyperpolarized by opioids were located in the more ventral areas of the ventrolateral periaqueductal gray.

8 These studies demonstrate that opioids acting on μ -receptors increase potassium conductance in a sub-population of large neurones located predominantly in the lateral column of the periaqueductal gray. The neurones hyperpolarized by opioids could be involved in the antinociceptive actions of opioids, but might also be involved in other functions because a large proportion lie outside of the main 'antinociceptive zone' of the periaqueductal gray. It is also unlikely that these neurones are GABAergic, suggesting that they might not participate in the postulated antinociceptive action of opioids mediated via disinhibition of neurones which project to the ventral medulla.

Keywords: Periaqueductal gray; opioid; enkephalin; μ -receptor; δ -receptor; κ -receptor; intracellular recording; potassium conductance; analgesia

Introduction

The periaqueductal gray (PAG) is an important site for the antinociceptive actions of opioids. Direct microinjections of morphine into PAG have been reported to produce antinociception, via an effect of neurones located in the rostral ventromedial medulla which participate in descending antinociceptive mechanisms (Yaksh *et al.*, 1988; Fields *et al.*, 1991). The rat PAG is rich in nerve terminals and fibres containing endogenous opioids (see Reichling *et al.*, 1988) and electrical stimulation in PAG has been reported to pro-

duce antinociception which can be antagonized by naloxone (Adams, 1976; Akil *et al.*, 1976). The PAG is also thought to be involved in the expression of opioid withdrawal signs in morphine-dependent animals (Laschka *et al.*, 1976; Bozarth & Wise, 1984; Maldonado *et al.*, 1992).

Opioid-mediated antinociception was most pronounced when micro-injections or electrical stimulation were directed at the caudal part of ventrolateral PAG (Yaksh *et al.*, 1988). This is consistent with the notion that the PAG is organised into anatomically and functionally distinct columns of neurones extending along the rostrocaudal axis (see Bandler *et al.*, 1991). However, little is known of the actions of

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opioids on membrane properties of PAG neurones, or actions on neurones in different functional subdivisions of the PAG.

From a study of extracellular recordings from brain slices, opioids were reported to excite, inhibit or have no effect on neurones distributed throughout the PAG (Behbehani *et al.*, 1990). By use of intracellular electrodes, neurones located in the dorsolateral PAG were shown to be inhibited by opioids (*ibid*). The present study used intracellular recordings from neurones in the lateral and ventrolateral PAG in brain slices to characterize the effects of opioids on membrane properties in subdivisions which are thought to be involved in antinociception. The accompanying paper considers the effects of opioids on synaptic transmission in PAG.

Disinhibition of neurones which project to the ventral medulla, mediated via inhibition of GABAergic interneurones by opioids, has been proposed as the mechanism by which opioids act in PAG to induce antinociception (Basbaum & Field, 1984). Similar mechanisms appear to account for excitatory effects of opioids elsewhere in the central nervous system. In many of these cases distinct morphological and/or physiological properties of opioid-sensitive (presumably GABAergic) and -insensitive neurones have been described (Madison & Nicoll, 1988; Lacey *et al.*, 1989; Pan *et al.*, 1990; Johnson & North, 1992). In the present study physiological properties of opioid-sensitive and -insensitive neurones in PAG were also characterized and morphological properties defined by use of intracellular labelling with biocytin.

Methods

Preparation of tissue and solutions

Male Sprague-Dawley rats (150–250 g) were anaesthetized with halothane, decapitated, and horizontal brain slices containing PAG (300 μ m) were cut and maintained in physiological saline at 35°C. Slices containing PAG lateral or ventral to the aqueduct were transferred to a tissue bath. Submerged slices were superfused with physiological saline at 35°C (1.5 ml min⁻¹). The physiological saline solution contained (in mM): NaCl 126, KCl 2.5, NaH₂PO₄ 1.2, MgCl₂ 1.2, CaCl₂ 2.4, glucose 11 and NaHCO₃ 24 and was gassed with 95% O₂ + 5% CO₂. Drugs were applied to the slice by changing the solution to one that differed only in its content of the drug.

Electrophysiological recording

Intracellular recordings of membrane potential were made with microelectrodes (70–120 M Ω , filled with 2 M KCl + 2% biocytin buffered with 50 mM Tris HCl, pH 7.4) using a single electrode current- and voltage-clamp amplifier (Axoclamp-2A). The approximate locations of impaled neurones were plotted on an atlas of the rat PAG (Paxinos & Watson, 1986). Recordings of membrane potential and applied current were plotted directly on chart recorder paper as well as being digitized for later analyses (PCLAMP and AXOTAPE software, Axon Instruments). Electrode resistance was monitored and balanced throughout experiments. For measurement of the amplitude of hyperpolarizations, membrane potential was held between -65 and -75 mV by application of constant current through the recording electrode. Input resistance was determined throughout with brief (250 ms) hyperpolarizing current pulses (0.3 Hz, -40 to -200 pA). The same currents were applied throughout drug applications. For determination of voltage-current relationships, membrane potential was monitored over a range (usually -50 to -150 mV) by applying an incremental series of current pulses of 200 ms duration. In these cases, the basal membrane potential was adjusted to control values during drug application by application of constant current. The total applied current was

used to determine voltage-current relationships. In several cases stable recordings were achieved with sufficiently low resistance electrodes (70–80 M Ω) to permit the use of discontinuous voltage-clamp at switching frequencies of 2–3 kHz while continuously monitoring the potential at the headstage with a separate oscilloscope. All data are expressed as mean $\pm$ s.e. mean.

Histochemistry

After recording, slices were fixed for at least 12 h in 10% formalin/phosphate buffer solution at 4°C. Slices were then rinsed in phosphate buffer and incubated in 0.3% H₂O₂/phosphate buffer (0.1 M pH 7.2) solution for 30 min. A further rinse was performed before incubating the slice in 0.3% Triton X-100/phosphate buffer solution for four days at 4°C. Slices were then rinsed and incubated in a solution containing 1:200 dilution of Extravidin peroxidase (Sigma, St Louis), and phosphate buffer for 2 h at room temperature with gentle rotations. This was followed by a rinse in 0.04% nickel/phosphate buffer solution for 1 h. The final incubation was in a solution containing 3,3'-diaminobenzidine (0.05%), nickel (0.04%), H₂O₂ (0.005%) and phosphate buffer. The reaction was stopped by rinsing the slice several times in phosphate buffer, when background staining started to become prominent. Slices were then mounted on gelatinised slides, dried, dehydrated in a graded series of alcohol and histolene, and coverslipped in D.P.X. mounting medium (BDH, UK). The coordinates of each stained section were identified from a rat brain atlas (Paxinos & Watson, 1986) and morphological properties were characterized as described in the results section. All morphological data are expressed as mean $\pm$ s.e. mean.

Drugs and reagents

Biocytin, Met-enkephalin and tetrodotoxin were obtained from Sigma (St Louis, U.S.A.), Tyr-D-Ala-Gly-MePhe-Glyol (DAMGO) and Tyr-D-Pen-Gly-Phe-D-Pen (Pen = penicillamine, DPDPE) from National Institute on Drug Abuse (U.S.A.), *trans*-(+)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrro-

Figure 1 Responses of PAG neurones to Met-enkephalin (30 μ M). (a) Example of a neurone hyperpolarized by Met-enkephalin. The hyperpolarization was associated with decreased input resistance (decreased amplitude of downward deflections). (b) Example of lack of effect of Met-enkephalin on membrane potential or input resistance. Downward deflections are membrane potential responses to constant current pulses passed through the recording electrode (-120 pA, 250 ms in a, -50 pA, 250 ms in b).

lidinyl)-cyclohexyl]benzeneacetamide methane sulphonate (U50488H) from Upjohn and naloxone hydrochloride from Research Biochemicals Inc (Natick, MA, U.S.A.).

Results

Opioids acting on μ -receptors directly hyperpolarize a sub-population of PAG neurones

Superfusion of Met-enkephalin (10–30 μ M) produced a hyperpolarization in 29% of PAG neurones (38/130). Hyperpolarizations were associated with a decrease in membrane resistance (Figure 1a, Figure 4). Met-enkephalin did not affect the membrane properties of the other 92 neurones tested (Figure 1b). The hyperpolarization induced by Met-enkephalin persisted in the presence of agents which block synaptic transmission ($n = 3$, 1 μ M tetrodotoxin or tetrodotoxin + 4 mM Co^{2+}), suggesting a direct action on impaired neurones. These agents completely abolished electrically-evoked postsynaptic potentials (see Chieng & Christie, 1994).

Neurones which were hyperpolarized by Met-enkephalin were also hyperpolarized by the selective μ -receptor agonist, DAMGO (e.g. Figure 2a, $n = 17$). The effects of DAMGO were completely reversed by the opioid antagonist, naloxone (1 μ M, $n = 3$, e.g. Figure 2b). Figure 3 depicts the concentration-response relationship of DAMGO. The EC_{50} was approximately 80 nM, and maximum response was achieved at approximately 3 μ M. When tested in the same neurones, the maximum amplitudes of the hyperpolarizations produced by DAMGO (8.5 ± 1.2 mV, $n = 17$) and Met-enkephalin (Figure 3, 7.7 ± 1.2 mV, $n = 17$) were similar (paired- $t = 2.0$, $P > 0.05$).

The membrane properties of neurones which were hyperpolarized by Met-enkephalin were not affected by selective δ - (DPDPE, 3 μ M, $n = 7$, Figure 2c) or κ -receptor (U50488H, 3 μ M, $n = 5$, Figure 2d) agonists. The solutions of DPDPE

and U50488H used in these experiments were subsequently found to be pharmacologically active at appropriate concentrations on δ - and κ -receptors in mouse vas deferens (N. Lavidis, personal communication) and guinea-pig ileum preparations (data not shown) respectively.

Opioids increase an inwardly rectifying potassium conductance

Opioids decreased input resistance at all potentials tested (Figures 4 and 5). For determination of voltage-current relationships (Figure 4), an incremental series of current pulses was applied through the recording electrode. The membrane potential during the application of Met-enkephalin or DAMGO was adjusted to the pre-drug level by application of constant current and the same series of current pulses was then applied. The hyperpolarization produced by opioids

Figure 3 Concentration-response curve of DAMGO in neurones which were hyperpolarized by Met-enkephalin. The effect of DAMGO is expressed on the ordinate scale as a percentage of the hyperpolarization produced by Met-enkephalin (10 or 30 μ M) in the same neurone. The EC_{50} for DAMGO was approximately 80 nM.

Figure 2 Hyperpolarizations induced by selective μ -, but not δ - or κ -receptor agonists. (a) DAMGO (3 μ M) induced a reversible hyperpolarization and decreased input resistance. (b) The hyperpolarization induced by DAMGO was completely antagonized by naloxone (1 μ M). The selective δ -receptor agonist DPDPE (3 μ M, c) and κ -receptor agonist U50488H (3 μ M, d) had no effect. These recordings (c and d) are from a single neurone which were hyperpolarized by Met-enkephalin (-5 mV at -71 mV). Drugs were superfused during periods indicated by solid bars. Resting membrane potentials of neurones were maintained at -71 mV. Downward deflections are membrane potential responses to constant current pulses passed through the recording electrode (-70 pA, 250 ms in a and b; -300 pA, 250 ms in c and d). Periods of superfusion of drugs are shown by bars. For abbreviations, see text.

Figure 4 Voltage-current relationships (raw data) of two neurones (one neurone in a and b, another in c and d) to opioids. Left figures (a and c) were sampled just prior to superfusion of opioids, and right figures (b and d) during opioid superfusion. Incremental step current pulses (60 pA increments from -270 pA, 200 ms in a and b, 70 pA from -350 pA in c and d) were applied for 200 ms (current record not shown). Resting membrane potential was maintained at -70 mV in each example by applying a constant current to the recording electrode (a = -10 pA, b = 80 pA, c = -70 pA, d = -50 pA). Resting (i.e., without superfusion of opioids) inward rectification (decreased membrane resistance at very negative membrane potentials) was evident in both neurones. DAMGO ($3 \mu\text{M}$, b) and Met-enkephalin ($30 \mu\text{M}$, d) hyperpolarized (compensated by application of constant current in record, see above) and decreased input resistance of each neurone at all potentials tested. The second neurone (c and d) displayed a slowly developing depolarization during large hyperpolarizing current pulses, which slowly relaxed on repolarization. Met-enkephalin had no effect on this slowly developing depolarization. Met-enkephalin and DAMGO did not substantially affect action potentials or afterhyperpolarizations in either neurone.

Figure 5 Effects of Met-enkephalin ($30 \mu\text{M}$) on subtracted steady-state current-voltage relationship of a single neurone in two extracellular potassium concentrations (control current subtracted from current in the presence of Met-enkephalin): (○) 2.5 mM , (●) 6.5 mM potassium.

(10 – $30 \mu\text{M}$ Met-enkephalin, $n = 10$, or $3 \mu\text{M}$ DAMGO, $n = 6$) reversed polarity at $-111 \pm 3 \text{ mV}$ ($n = 16$, Figures 4 and 5). Using voltage-clamp, the reversal potential shifted from -110 mV to -86 mV when the extracellular potassium concentration was raised from 2.5 mM to 6.5 mM (Figure 5). This 24 mV shift is in close agreement with the value predicted by the Nernst equation (25 mV) for a potassium conductance. In this experiment, the slope conductance of the opioid-induced current showed inward rectification both in 2.5 mM (1.1 nS at -70 mV and 2.3 nS at -120 mV) and

6.5 mM extracellular potassium (1.8 nS at -70 mV and 10.7 nS at -90 mV). Complementary results were obtained from recordings of membrane potential. The opioid-induced decrease in input resistance was smaller when determined between -70 and -80 mV ($17 \pm 4\%$, $n = 16$) than between -110 and -140 mV ($37 \pm 6\%$, $n = 16$, difference = $20 \pm 6\%$, paired $t = 3.37$, $P < 0.005$). Other membrane properties including amplitude and duration of action potentials and afterhyperpolarizations, and the slowly developing depolarization at negative potentials were not affected (Figure 4).

Other membrane properties of opioid-sensitive and -insensitive neurones

In the absence of applied opioids, opioid-sensitive neurones displayed more resting inward rectification than -insensitive neurones. The input resistance of opioid-sensitive neurones was significantly smaller at more negative membrane potentials (-110 to -140 mV , $147 \pm 18 \text{ M}\Omega$, $n = 19$) than between -70 and -80 mV ($288 \pm 36 \text{ M}\Omega$, difference = $140 \pm 31 \text{ M}\Omega$, paired $t = 4.59$, $P < 0.001$). Input resistance of opioid-insensitive neurones was also smaller at negative membrane potentials (-110 to -140 mV , $248 \pm 13 \text{ M}\Omega$, $n = 67$) than between -70 and -80 mV (289 ± 13 , difference = $47 \pm 5 \text{ M}\Omega$, paired $t = 6.00$, $P < 0.0001$). However, the decrease in input resistance at negative potentials was significantly larger in opioid-sensitive than -insensitive neurones ($40 \pm 6\%$, $n = 19$ versus $17 \pm 2\%$, $n = 67$, unpaired $t = 4.9$, $P < 0.001$).

With the exception of the amplitude of afterhyperpolariza-

Figure 6 Examples of three morphological types of biocytin-filled PAG neurones. (a) Fusiform, (b) triangular and (c) multipolar, from horizontal midbrain sections. The somata are ovoid shaped and primary dendrites arise from thick extensions of somata. Dendrites branch out in a radiating pattern. Note varicosities, as well as isolated and sparse dendritic spines. Scale bar = 50 μ m.

tions, other membrane properties of opioid-sensitive and -insensitive neurones did not differ significantly. The amplitude of afterhyperpolarization was 20 ± 2 mV ($n = 16$) in opioid-sensitive and 25 ± 1 mV ($n = 58$, unpaired $t = 2.45$, $P < 0.02$). Sixty-three percent (12/19) of opioid-sensitive neurones fired spontaneous action potentials at 10 ± 4 Hz (range 0.2 to 50 Hz) in the absence of applied current, versus 50% (34/68) of opioid-insensitive neurones (4 ± 1 Hz, range 0.2 to 14 Hz). Resting membrane potential was -66 ± 4 mV ($n = 7$) in quiescent opioid-sensitive neurones (versus -71 ± 2 mV in opioid-insensitive neurones, $n = 32$). Action potentials had a threshold of -56 ± 1 mV ($n = 16$) in opioid-sensitive neurones, versus -54 ± 1 mV ($n = 58$) in opioid-insensitive neurones, amplitude of 62 ± 2 mV (versus 63 ± 1 mV), duration at threshold of 1.1 ± 0.1 ms (versus 1.4 ± 0.1 ms) and 10–90% rise-time 0.27 ± 0.02 ms (versus 0.31 ± 0.01 ms). During large hyperpolarizing current pulses, a

Figure 7 Anatomical locations of opioid-sensitive (●) and -insensitive (○) neurones in four dorso-ventral levels of horizontal PAG sections (5.1, 5.6, 6.1 mm and 6.6 mm ventral to bregma). The precise locations of neurones were determined by visualization of biocytin staining and plotted on diagrams prepared from a stereotaxic atlas of the rat brain (Paxinos & Watson, 1986). Abbreviations: 4v, fourth ventricle; aq, aqueduct; DR, dorsal raphe nucleus; ldtg, laterodorsal tegmental nucleus.

slowly developing depolarization which slowly relaxed on repolarization occurred in 21% (4/19) of opioid-sensitive neurones and 8% (5/65) of opioid-insensitive neurones (e.g. Figure 4c,d). The membrane time constant of opioid-sensitive neurones was 22 ± 2 ms ($n = 19$) between -60 and -80 mV (versus 26 ± 1 ms, $n = 67$ for opioid-insensitive neurones).

Morphology of opioid-sensitive and -insensitive neurones

No significant morphological differences were observed between opioid-sensitive and -insensitive neurones (Figure 6). Soma diameters (measured along the longest axis) of opioid-sensitive neurones (22 ± 1 μ m, range 13 to 30 μ m, $n = 20$) were nearly identical to opioid-insensitive neurones (22 ± 1 μ m, range 10 to 33 μ m, $n = 48$). Of the opioid-sensitive neurones, 58% were multipolar (11/19) with ovoid shaped soma (versus 49% [21/43] of opioid-insensitive neurones); 37% were triangular (versus 30% of opioid-insensitive neurones) and 5% fusiform (versus 21% of opioid-insensitive neurones). Orientations of the somata were mostly in the horizontal plane (16/21 versus 37/40 opioid-insensitive neurones). Dendritic spread was usually in a radiating pat-

tern in all directions. Primary dendrites of opioid-sensitive and -insensitive neurones arose from thick extensions of the soma and formed a number of branches (mode = 4 for opioid-sensitive and 3 for -insensitive neurones). Of opioid-sensitive neurones, 29% (6/21) had spines on the primary dendrites (versus 56% [23/41] of opioid-insensitive neurones) and 10% had varicosities (versus 26% of opioid-insensitive neurones). The length of the primary dendrites was $33 \pm 10 \mu\text{m}$ ($n = 21$) with a diameter $2.5 \pm 0.2 \mu\text{m}$ ($n = 21$) (versus 45 ± 8 , and 2.7 ± 0.1 , $n = 47$ respectively for opioid-insensitive neurones). Secondary dendrites were usually very long ($155 \pm 26 \mu\text{m}$, $n = 21$) and thin ($1.3 \pm 0.1 \mu\text{m}$, $n = 21$) (versus $113 \pm 15 \mu\text{m}$ and $1.4 \pm 0.1 \mu\text{m}$, $n = 44$ respectively for opioid-insensitive neurones). Spines were visible on 71% of secondary dendrites, and 67% had varicosities (versus 72% and 49% respectively for opioid-insensitive neurones). In all cases spines and varicosities were isolated and sparse (Figure 6). Tertiary dendrites usually appeared to have been damaged during slice preparation. Where they could be identified, axons ($<0.5 \mu\text{m}$ diameter) were usually branched with arborisations oriented in many directions. Axons appeared to originate from the soma in 38% of opioid-sensitive neurones (3/8 versus 73% [24/33] for opioid-insensitive neurones), and from primary dendrites in others.

Anatomical location of opioid-sensitive and -insensitive neurones

Figure 7 depicts the anatomical location of opioid-sensitive and -insensitive neurones in horizontal sections of PAG. The proportions of neurones hyperpolarized by opioids significantly differed among the three main dorso-ventral levels categorized (5.1, 5.6 and 6.1 mm ventral to bregma, $\chi^2 = 29$, 2 d.f., $P < 0.001$). All neurones impaled in the most dorsal slices (lateral PAG, approximately 5.1 mm ventral to bregma) were hyperpolarized by opioids (9/9). In intermediate slices 50% of neurones (15/30) were hyperpolarized by opioids. At this dorso-ventral location the majority of opioid-sensitive neurones appeared to lie adjacent to the dorsal raphe nucleus. In more ventral slices (ventrolateral PAG, approximately 6.1 mm ventral to bregma), only 19% (14/75) of neurones scattered throughout the region were hyperpolarized by opioids. Although 20% (1/5) of neurones in the most ventral section (6.6 mm ventral to bregma) were also hyperpolarized by opioids, too few neurones were impaled for reliable comparisons to be made with other sections.

Discussion

The present study demonstrated that opioid agonists selective for μ -receptors directly hyperpolarized a sub-population of PAG neurones by increasing an inwardly rectifying potassium conductance. Opioid-sensitive neurones were impaled more frequently in lateral than in ventrolateral PAG. With the exception of greater resting inward rectification and smaller amplitude of afterhyperpolarizations in opioid-sensitive neurones, other membrane and morphological properties did not discriminate the sub-populations.

Opioids directly hyperpolarized PAG neurones by increasing an inwardly rectifying potassium conductance. The hyperpolarization reversed polarity at the expected equilibrium potential for potassium ions (E_K), assuming an intracellular potassium concentration of 165 mM. The reversal potential became less negative in close agreement with the Nernst equation when the external potassium concentration was increased, strongly suggesting that the hyperpolarization was due to increased potassium conductance. The opioid-induced potassium conductance displayed inward rectification at hyperpolarized potentials, i.e. the change in input resistance (or conductance using voltage-clamp) was greater at potentials negative to E_K than at more positive potentials. A similar mechanism of action of opioids has been described

in a variety of central and peripheral neurones (e.g. Williams *et al.*, 1982; North *et al.*, 1987; Christie & North, 1988; Madison & Nicoll, 1988; Lacey *et al.*, 1989; Pan *et al.*, 1990; Johnson & North, 1992).

The opioid-induced hyperpolarization of PAG neurones was due to activation of μ -receptors. The μ -receptor selective agonist, DAMGO, produced hyperpolarizations of similar amplitude to Met-enkephalin, whilst δ - and κ -receptor selective agonists had no effect. The effects of DAMGO and Met-enkephalin were fully antagonized by naloxone. Furthermore, the potency of DAMGO was similar to that previously reported for μ -receptor actions in central neurones (e.g. Christie & North, 1988). Inhibition of synaptic transmission by opioids was also largely mediated by μ -receptors in PAG (Chieng & Christie, 1994). These results agree with reports of a moderate (Mansour *et al.*, 1987; Tempel & Zukin, 1987) to high (Waksman *et al.*, 1986) density of μ -receptor binding sites, but not δ -receptor binding sites (Waksman *et al.*, 1986; Mansour *et al.*, 1987; Tempel & Zukin, 1987) in rat PAG. The μ -receptor-mediated effects observed in the present study are also generally compatible with the strong antinociception induced by microinjections of opioids into the PAG (e.g. Yaksh *et al.*, 1988), and induction of withdrawal behaviour by microinjections of opioid antagonists into PAG of morphine-dependent rats (e.g. Maldonado *et al.*, 1992), or by chronic infusion of morphine into PAG (Bozarth & Wise, 1984). However, no direct effects of κ -receptor agonists were observed in the present study despite reports of a high density of κ -receptor binding sites in PAG (Mansour *et al.*, 1987; Tempel & Zukin, 1987).

Opioids directly hyperpolarized a sub-population of neurones in PAG. The reasons for more pronounced resting inward rectification and smaller afterhyperpolarizations in opioid-sensitive neurones are unclear at present, but might suggest that other receptors and/or ion channels in addition to μ -receptors differ between sub-populations. It might be argued that neurones were artefactually classified as opioid-insensitive in the present study, perhaps due to poor quality of impalements. However, this is unlikely because only impalements considered to be of high quality were used (see methods), and most other physiological properties did not differ between sub-populations. Furthermore, 100% ($n = 16$) of the opioid-insensitive neurones were hyperpolarized by the GABA_B-receptor agonist, baclofen (Chieng & Christie, unpublished observation), which presumably acts via a similar intracellular mechanism to μ -receptor agonists (e.g. Christie & North, 1988) and suggests that second messenger systems were functional in opioid-insensitive neurones.

Populations of opioid-sensitive and -insensitive neurones studied in other brain regions were easily discriminated on the basis of physiological and/or morphological properties, e.g. in the hippocampus (Madison & Nicoll, 1988), substantia nigra (Lacey *et al.*, 1989), ventral tegmental area (Johnson & North, 1992) and nucleus raphe magnus (Pan *et al.*, 1990). However, with the exception of significantly greater resting inward rectification and smaller afterhyperpolarizations in opioid-sensitive neurones, other physiological and morphological features of PAG neurones failed to distinguish separate sub-populations. Glutamatergic and GABAergic components of spontaneous and electrically-evoked synaptic potentials also failed to discriminate the two sub-populations (Chieng & Christie, 1994). Membrane properties of neurones were similar to those previously described for guinea-pig PAG neurones (Sanchez & Ribas, 1991).

It is not clear whether the relatively uniform properties of opioid-sensitive and -insensitive neurones observed in the present study suggest a novel organisation of opioid-sensitive and -insensitive neurones in PAG, or are perhaps due to biased sampling of large cells by intracellular recording techniques. The mean soma diameter of impaled neurones were $22 \mu\text{m}$ and very few small ($<15 \mu\text{m}$ diameter) neurones were sampled. In contrast, the mean diameter was reported to be $14 \mu\text{m}$ in Nissl and Golgi staining studies of rat lateral and

ventrolateral PAG (Beitz, 1985; Beitz & Shepard, 1985). Only 33% of Golgi-stained neurones correspond the large fusiform, triangular, and multipolar types impaled in the present study (Beitz & Shepard, 1985). Barbaresi & Manfrini (1988) also reported that most glutamate decarboxylase immunoreactive interneurons in lateral and ventrolateral PAG were small (mean diameter of 10 μ m calculated from reported area). It is therefore possible that opioids also act on physiologically distinct small neurones. It is also possible that δ - and κ -receptors on small neurones mediate some actions of opioids in PAG.

The anatomical locations of the two types of PAG neurones found in the present study support the concept that PAG is organised into functional columns extending along the rostrocaudal axis (see Bandler *et al.*, 1991). Opioids hyperpolarized a greater proportion of neurones located in the lateral column (100% of neurones) and dorsal parts of the ventrolateral column (50% of neurones) than in the ventrolateral PAG (19% of neurones, Figure 7). Behbehani *et al.* (1990) also reported that Met-enkephalin hyperpolarized 95% of neurones located in dorsolateral PAG. In contrast, the main antinociceptive zone identified by microinjections of opioids or electrical stimulation was reported to lie within the caudal ventrolateral region of PAG (Yaksh *et al.*, 1988). The somata of neurones which were hyperpolarised by opioids therefore appear to lie outside of the main antinociceptive zone of PAG. However, this discrepancy should not be viewed as critical. Firstly, it is possible that most of the opioid-sensitive neurones sampled in this study have nothing to do with eliciting antinociception, i.e. they might not interact with projection neurones which are parts of the descending antinociceptive pathway. Our physiological roles of PAG influenced by opioids might include organis-

ation of autonomic and somatic components of defence and escape behaviours, or perhaps cardio-respiratory functions (see Bandler *et al.*, 1991). Second, it is conceivable that the abundant small neurones which were not sampled in the ventrolateral PAG (see above) do respond to opioids and are involved in antinociception. Finally, it should be noted that inhibition of synaptic transmission by opioids occurred throughout the lateral and ventrolateral PAG and could be important for the antinociceptive actions of opioids (Chiang & Christie, 1994).

Disinhibition of neurones which project to the ventral medulla was proposed as the mechanism by which opioids act in PAG to induce antinociception (Basbaum & Fields, 1984). This has been postulated to occur via opioid inhibition of tonically active GABAergic interneurons, thereby disinhibiting PAG output neurones projecting to the ventral medulla. Although neurones which were hyperpolarized by opioids in the present study could conceivably participate in this kind of disinhibition, it is unlikely that they were GABAergic because neurones in PAG reported to be immunoreactive for glutamate decarboxylase (Barbaresi & Manfrini, 1988) were much smaller than the neurones sampled in the present study. The neurotransmitters present and anatomical organisation of opioid-sensitive and -insensitive neurones in the PAG have yet to be directly established.

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