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Hyperpolarization by $GABA_B$ receptor agonists in mid-brain periaqueductal gray neurones *in vitro*

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1 The effects of $GABA_B$ receptor stimulation on membrane properties of rat periaqueductal gray neurones were studied by use of intracellular recordings from single neurones in superfused brain slices. Intracellular staining with biocytin was used to characterize the anatomical location of each impaled neurone.

2 The $GABA_B$ receptor agonist, baclofen, directly hyperpolarized or produced an outward current (single electrode voltage-clamp) in all 66 neurones tested. Baclofen-induced hyperpolarizations were concentration-dependent with an EC_{50} of approximately 0.6 μM and maximum hyperpolarization with 10 μM baclofen. Hyperpolarizations persisted in the presence of tetrodotoxin (1 μM, $n=2$).

3 2-OH-saclofen, a selective $GABA_B$ receptor antagonist, competitively antagonized baclofen-induced hyperpolarizations ($n=4$) with equilibrium dissociation constants estimated in two neurones to be 6 and 23 μM. Naloxone (1 μM) did not prevent hyperpolarizations induced by baclofen ($n=34$).

4 Hyperpolarizations induced by baclofen were associated with an increased inwardly rectifying potassium conductance. Ba^{2+} superfusion (5 to 10 mM) blocked this conductance increase ($n=4$). Elevation of extracellular potassium concentration (from 2.5 to 6.5 mM) shifted the reversal potential in agreement with predictions of the Nernst equation.

5 Hyperpolarizations produced by baclofen (10 μM) desensitized (>5% inhibition of the maximum response) in 7/22 neurones during continuous superfusion for 5 min. Strong desensitization (>25% inhibition of the maximum response) was observed in only 2/22 neurones in the ventrolateral periaqueductal gray. In contrast 6/9 neurones in the laterodorsal tegmental nucleus displayed strong desensitization.

6 These studies demonstrate that baclofen acting on $GABA_B$ receptors increases potassium conductance in all lateral and ventrolateral periaqueductal gray neurones. The neurones hyperpolarized by baclofen are likely to be involved in the well-established antinociceptive actions of baclofen in the ventrolateral periaqueductal gray, but might also be involved in other functions because many of them lie outside the main 'antinociceptive' zone of this region. The cellular mechanisms underlying baclofen-induced antinociception presumably differ from the postulated antinociceptive action of opioids, thought to be mediated via disinhibition of periaqueductal gray neurones which project to the ventromedial medulla.

Keywords: Periaqueductal gray; $GABA_B$ receptor; baclofen; 2-OH-saclofen; antinociception; opioids; desensitization; intracellular recording; laterodorsal tegmentum

Introduction

The mid-brain periaqueductal gray (PAG) is thought to play a role in integration of somatic and autonomic components of defence and escape behaviours, and cardio-respiratory functions (see Lovick, 1992; Bandler & Shipley, 1994). The PAG is also widely recognized as an integral part of a descending antinociceptive pathway (e.g. Fields *et al.*, 1988; 1991). The ventrolateral subdivision of PAG was found to induce antinociception most effectively both from intracerebral focal electrical stimulation (Fardin *et al.*, 1984a, b) and localized opioid injection (Yaksh *et al.*, 1976) studies. Hence, the ventrolateral PAG has been described as a 'pure' antinociceptive zone (Fardin *et al.*, 1984a, b).

Baclofen, a $GABA_B$ receptor agonist, has been reported to produce antinociception in animals (e.g. Cutting & Jordan, 1975; Levy & Proudfit, 1977; 1979; Proudfit & Levy, 1978) and analgesia in human subjects (Pinto *et al.*, 1972; Corli *et al.*, 1984; Fromm *et al.*, 1984). Localized injections of baclofen into the ventrolateral subdivision of PAG has also been reported to produce profound antinociception (Levy & Proudfit, 1979), but baclofen administration into other parts of PAG appeared to be less effective. The mechanisms underlying opioid-mediated antinociception in the ventrolateral PAG have been well studied, but the mechanisms of $GABA_B$ receptor-mediated antinociception have not. PAG contains moderate levels of $GABA_B$ binding sites (Bowery *et al.*, 1987; Chu *et al.*, 1990) and the distribution is quite even throughout the caudal PAG but with a slightly larger density of binding sites in the dorsal portion.

The cellular effects of activating $GABA_B$ receptors have been reported in a number of regions of the central nervous system (Newberry & Nicoll, 1985; Howe *et al.*, 1987; Osmanovic & Shefner, 1988; Christie & North, 1988; Colmers & Williams, 1988; Lacey *et al.*, 1988; Brooks *et al.*, 1992). Stimulation of postsynaptic $GABA_B$ receptors invariably inhibited neurotransmission and hyperpolarized neurones as a result of an increase in potassium conductance (Newberry & Nicoll, 1985; Howe *et al.*, 1987; Osmanovic & Shefner, 1988; Christie & North, 1988; Colmers & Williams, 1988; Lacey *et al.*, 1988; Brooks *et al.*, 1992). $GABA_B$ receptor activation has also been found to inhibit calcium currents in other cells (Bowery, 1989). The present study investigated the distribution and cellular mechanisms of $GABA_B$ receptor activation in the caudal lateral and ventrolateral PAG.

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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 μm) 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 and superfused submerged with physiological saline at 35°C (1.5 ml min^{-1}). The physiological saline solution (pH 7.4) contained (in mM) NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.4, glucose 11 and $NaHCO_3$ 24, and was gassed with 95% O_2 + 5% CO_2. 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 MΩ, 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) as previously described. The approximate locations of impaled neurones were plotted from 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. Only cells which had amplitude of action potential at least 50 mV were used for data analysis. 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 (0.2 to 0.3 Hz, –40 to –200 pA). The same current pulses 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 the pre-drug value by application of constant current; the total applied current was used to construct voltage-current relationships. Input resistance changes were determined from voltage-current plots by drawing tangents to the curves between –70 and –75 mV, and between –105 and –130 mV. In several cases stable recordings were achieved with sufficiently low resistance electrodes (70–80 MΩ) 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 ± 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% H_2O_2/phosphate buffer (0.1 M pH 7.2) solution for 30 min. A further rinse was performed before incubating slices 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, U.S.A.) and phosphate buffer for 2 h at room temperature with gentle rotations. This was followed by a rinse in 0.04% nickel ammonium sulphate/phosphate buffer solution for 1 h. The final incubation was in a solution containing 3,3'-diaminobenzidine (0.05%), nickel (0.04%), H_2O_2 (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 onto gelatinised slides, dried, dehydrated in a graded series of ethanol 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).

Drugs and reagents

Biocytin, baclofen, Met-enkephalin and tetrodotoxin were obtained from Sigma (St Louis, U.S.A.), naloxone hydrochloride from Research Biochemicals Incorporated (Natick, MA, USA), and 2-OH-saclofen was a gift from Dr J. Ong.

Results

Properties of PAG neurones were similar to those previously described (Chieng & Christie, 1994a). PAG neurones had action potential amplitude of 63 ± 1 mV (n = 66) and duration at threshold of 1.22 ± 0.1 ms (n = 66), with an after-hyperpolarization amplitude of 24 ± 1 mV (n = 62). Input resistance was 254 ± 14 MΩ (n = 63). Of the cells, 40% (25/62) were spontaneously active in the absence of applied current, with an action potential frequency of 5 ± 1 Hz (n = 25). The remaining quiescent cells had a resting membrane potential of –70 ± 1 mV (n = 37). Of biocytin stained cells, 64% (35/55) were multipolar, 31% were triangular and 5% were fusiform. The soma diameter of these neurones was 21 ± 1 μm (n = 55). Properties of nine laterodorsal tegmental nucleus neurones were not included in the present analyses (see Figure 5, below).

$GABA_B$ receptor activation caused a hyperpolarization

Baclofen produced a hyperpolarization (n = 63; Figure 1), or an outward current (voltage-clamp n = 3), in all PAG neurones tested. Hyperpolarizations were accompanied by decreased membrane resistance (Figure 1 and also see below). The effects persisted in the presence of tetrodotoxin (1 μM, n = 2; data not shown). The amplitude of hyperpolarizations was dependent on the concentration of baclofen applied, with a maximum response (14.6 ± 0.5 mV, n = 63, range 7 to 25 mV) produced by 10 or 30 μM baclofen (Figure 2). The EC_{50} was approximately 0.6 μM.

2-OH-saclofen antagonized the hyperpolarization induced by baclofen in all neurones tested (n = 4; Figure 3). From

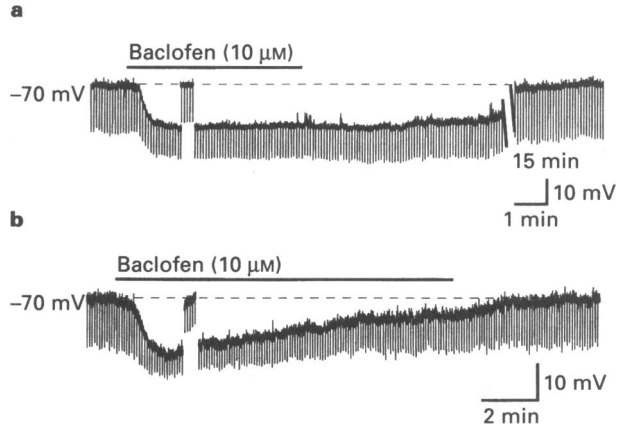

Figure 1 Baclofen-induced hyperpolarizations in two PAG neurones. The hyperpolarizations were simultaneously accompanied by a decreased input resistance of the cells (decreased amplitude of downward deflections, particularly when resting membrane potential was restored by direct current application). (a) Example of a neurone which did not display a diminished hyperpolarization upon continuous superfusion of baclofen (10 μM). (b) Example of a neurone which showed strong desensitization of the hyperpolarization upon continuous superfusion of baclofen (10 μM). Downward deflections are membrane potential responses to constant current pulses passed through the recording electrode (–40 pA, 200 ms, 0.2 Hz in (a), –80 pA, 250 ms, 0.2 Hz in (b)).

Schild analysis of one cell, the pA_2 was estimated to be 5.2, corresponding to K_c of 6 μM, with a slope of 0.83 (Figure 3). In another cell, 2-OH-saclofen (10 μM) shifted the concentration-response curve 1.4 fold to the right with an estimated K_c of 23 μM.

In neurones which were also hyperpolarized by Met-enkephalin (10 or 30 μM), baclofen (10 μM) produced a significantly larger maximum amplitude of hyperpolarization than Met-enkephalin (14.5±0.9 mV vs 10.0±1.9 mV, paired t test = 2.337, $P<0.05$, $n=11$). Baclofen-induced hyperpolarizations did not significantly differ between opioid-sensitive and -insensitive neurones (14.5±0.9 mV, $n=11$, opioid-sensitive versus 14.7±0.7 mV, $n=33$, opioid-insensitive, unpaired t test = 0.12, $P>0.05$, see Chieng & Christie, 1994a). Baclofen-induced hyperpolarizations were not significantly affected by superfusion with naloxone (1 μM, $n=34$).

Baclofen had little or no effect on other membrane properties of PAG neurones. Small, significant effects were observed on the duration of action potentials (1.4±0.1 ms vs 1.3±0.1 ms at threshold in baclofen, $P<0.01$, paired t test, $n=11$) and after hyperpolarizations (5.1±0.5 ms vs 4.3± 0.4 ms at half-maximal amplitude, $P<0.05$, paired t test, $n=11$). No significant effects were observed on action potential amplitude (65±2 mV vs 64±2 mV, $n=11$) and rise-time (0.28±0.01 ms vs 0.27±0.01 ms, $n=11$), nor on the amplitude (19±2 mV vs 18±2 mV, $n=11$) of afterhyperpolarizations.

GABA$_B$ receptor activation caused an increase in potassium conductance

From single electrode voltage-clamp recordings, the mean amplitude of current produced by 10 μM baclofen was 105±20 pA ($n=3$) at holding potentials of –62 to –65 mV. In current-clamp experiments, baclofen induced a decrease in input resistance over the entire range of membrane potentials sampled (–70 to –140 mV; Figure 4a). This baclofen-induced decrease in input resistance was smaller when determined between –70 and –75 mV (28±2%, $n=49$) than between –105

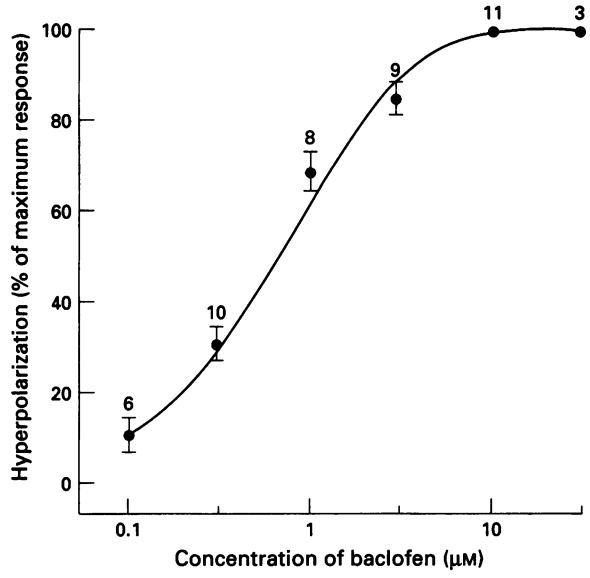

Figure 2 Concentration-response curve of the baclofen-induced hyperpolarization. The amplitude of the hyperpolarization on the ordinate scale is expressed as a percentage of maximum hyperpolarization produced by baclofen (10 or 30 μM) in the same neurone. The curve was fitted by eye. The EC_{50} for baclofen was approximately 0.6 μM.

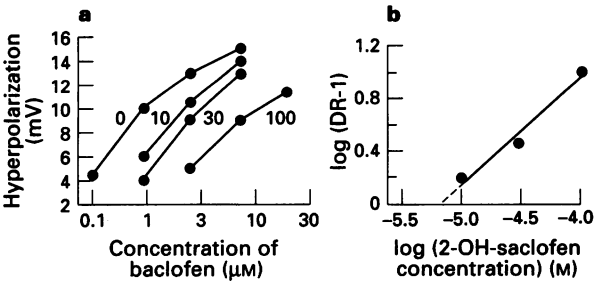

Figure 3 2-OH-saclofen antagonism of the hyperpolarization induced by baclofen in a single PAG neurone. (a) Increasing concentrations of 2-OH-saclofen (10, 30 and 100 μM as indicated) produced rightward shifts of the concentration-response curves. (b) Schild analysis of the data yielded a pA_2 for 2-OH-saclofen of 5.2 (slope is 0.83, least-squares linear regression).

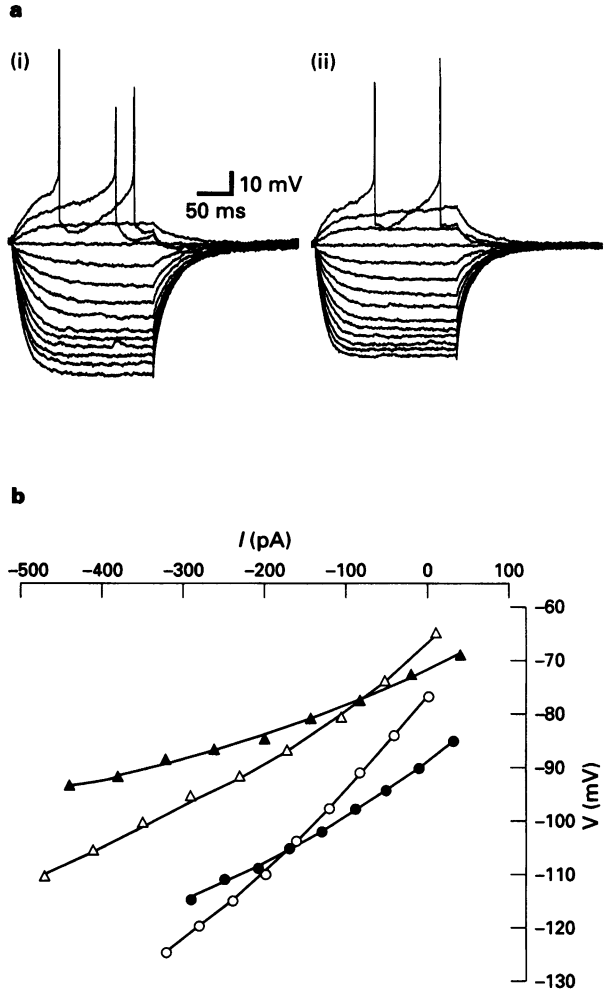

Figure 4 (a) Voltage-current relationships (raw data) obtained from a PAG neurone in response to baclofen (10 μM). Left figure (i) was sampled just prior to superfusion of baclofen, and right figure (ii) during baclofen superfusion. Incremental step current pulses (25 pA increments in both i and ii) were applied for 200 ms (current record not shown). Resting membrane potential was maintained at –70 mV by applying a constant current to the recording electrode (i: I = –10 pA; ii: I = 30 pA). The response to baclofen did not desensitize during sampling of the voltage-current relationship. (b) Voltage-current relationships of a single PAG neurone in two extracellular potassium concentrations (2.5 mM (○,●) and 6.5 mM (▽,▲)), prior to (open symbols) and during superfusion of baclofen (10 μM, solid symbols). The reversal potentials of the responses to baclofen in 2.5 and 6.5 mM extracellular potassium were –106 and –78 mV, respectively.

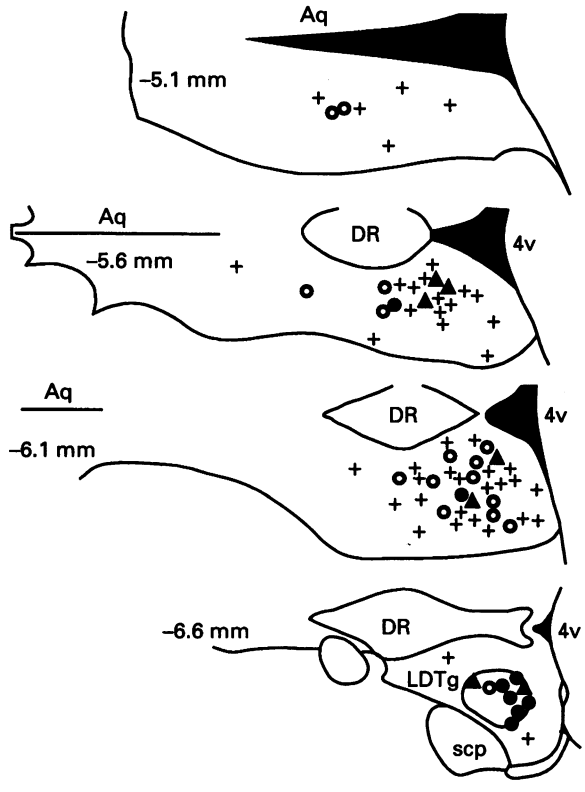

Figure 5 Locations of individual baclofen-responsive neurones (desensitizing, non-desensitizing and others) in four dorso-ventral levels of horizontal PAG sections (5.1, 5.6, 6.1 and 6.6 mm ventral to bregma): (○) represent neurones which did not show desensitization during 5 min continuous superfusion of baclofen; (▲) represent neurones which showed a small desensitization during 5 min continuous superfusion of baclofen (10 μM); (●) represent neurones which showed a strong desensitization (>25% reduction of hyperpolarization) during 5 min continuous superfusion of baclofen; (+) represent neurones in which baclofen was applied for less than 5 min and hence are indeterminate with regard to desensitization. A high concentration of baclofen (10 or 30 μM) was used in each case. Abbreviations: 4v, fourth ventricle; Aq, aqueduct; DR, dorsal raphe nucleus; LDTg, laterodorsal tegmental nucleus; scp, superior cerebellar peduncle.

and −130 mV (41±2%, $n=49$, difference = 14±1%, paired t test = 7.2, $P<0.0001$). These results indicate that the degree of inward rectification evident in Figures 4a and 4b was increased in the presence of baclofen.

The baclofen-induced hyperpolarization reversed polarity near the expected potassium equilibrium potential (−107± 1 mV, $n=52$ including 3 voltage-clamp experiments, Figure 4b). The reversal potential shifted from −107±3 mV to −80± 1 mV ($n=3$) when the extracellular K^+ concentration was increased from 2.5 to 6.5 mM (Figure 4b). This 27 mV increase was in close agreement with Nernst equation (25 mV). The baclofen-induced hyperpolarization was blocked by superfusion with Ba^{2+} (5 to 10 mM, $n=4$; data not shown).

Acute desensitization of baclofen-induced hyperpolarizations

In 32% (7/22) of PAG neurones, acute desensitization of the hyperpolarization was observed within 5 min of continuous application of high concentrations of baclofen (10 or 30 μM; Figure 1); 5/22 neurones showed a small acute desensitization (13.9±0.9% reduction of the maximum hyperpolarization, $n=5$), and 2/22 neurones displayed a strong acute desensitization (79% and 38%, $n=2$). The distribution of all impaled neurones, including those displaying acute desensitization, is plotted in Figure 5. It is interesting to note that a substantial proportion (6/9) of neurones located within the laterodorsal tegmental nucleus showed a strong acute desensitization of the response to baclofen (55±5%, $n=6$; not included in other analyses; Figure 5).

Discussion

The present study demonstrated that activation of post-synaptic GABA$_B$ receptors hyperpolarized all PAG neurones tested in the lateral and ventrolateral subdivisions of PAG as a result of an increased inwardly rectifying potassium conductance. The estimated values of EC_{50} of baclofen (0.6 μM) and pA_2 of 2-OH-saclofen (5.2) determined here were comparable to those found in other studies. The EC_{50} of baclofen was reported to be 1.3 μM in substantia nigra (Lacey *et al.*, 1988), 1.5 μM in dorsal raphe nucleus (Colmers & Williams, 1988) and 2.0 μM in locus coeruleus (Osmanovic & Shefner, 1988). The pA_2 reported for 2-OH-saclofen was approximately 5 in guinea-pig ileum and vas deferens, and rat cortical slices (Kerr *et al.*, 1988; 1989). Although not very potent, 2-OH-saclofen competitively antagonized the response to baclofen, indicating a GABA$_B$ receptor-mediated action. These results indicated that stimulation of GABA$_B$ receptors in PAG operated through a cellular mechanism similar to that found in other central neurones (Newberry & Nicoll, 1985; Howe *et al.*, 1987; Osmanovic & Shefner, 1988; Christie & North, 1988; Colmers & Williams, 1988; Lacey *et al.*, 1988; Brooks *et al.*, 1992).

Although the present study did not directly address potential inhibition of Ca^{2+}-currents in PAG neurones, only small effects were observed on duration of action potentials and afterhyperpolarizations, both of which are at least partially dependent on Ca^{2+} influx (unpublished observations and Sanchez & Ribas, 1991). It is not clear whether the small effects noted here resulted from reduced Ca^{2+} influx, or were secondary to the increased membrane conductance produced by baclofen.

The observation that all PAG neurones tested in the present study were hyperpolarized by baclofen may question the general validity of currently accepted models of the role of this brain region in mechanisms of antinociception. Disinhibition of neurones which project to the ventral medulla has been proposed as the mechanism by which opioids act in PAG to produce antinociception (Basbaum & Fields, 1984; Fields *et al.*, 1991). This has been postulated to occur via opioid inhibition of tonically active GABAergic interneurones in PAG, thereby disinhibiting output neurones which project to the ventral medulla. Baclofen microinjections into the 'antinociceptive' zone in the ventrolateral PAG produce profound antinociception (Levy & Proudfit, 1979), but all neurones in this region, presumably including projection neurones, were strongly hyperpolarized and therefore inhibited by baclofen in the present study. It might be argued that the present study has not directly demonstrated inhibition of projection neurones by baclofen. However, it seems unlikely we failed to sample any projection neurones because more than 18% of neurones throughout the PAG are thought to project to the ventral medulla (Reichling & Basbaum, 1990). Moreover, projection neurones are concentrated in the ventrolateral subdivision and are of a similar size to those sampled in the present study. Although the present results appear to suggest that baclofen would inhibit PAG projection neurones *in vivo*, the possibility that the net effect of baclofen administration *in vivo* is to reduce inhibitory synaptic activity to a greater extent than direct inhibition of projection neurones cannot be ruled out. Our previous studies also suggested that the actions of opioids in PAG are more complex than suggested by a model of disinhibition. Opioid agonists inhibited GABAergic and glutamatergic synaptic potentials nearly equally throughout the PAG (Chieng & Christie, 1994b), but simple models of disinhibition would predict predominantly GABAergic inhibition. The majority of neurones directly hyperpolarized by opioids were

located in the lateral rather than ventrolateral PAG (Chieng & Christie, 1994a). The lateral PAG is not thought to be involved in opioid antinociception (Yaksh *et al.*, 1976), so the functional significance of this opioid action in not yet clear.

In general, baclofen- and opioid-mediated antinociception appear to result from actions on distinct but overlapping neural circuits. For example, both are antinociceptive in the spinal cord. In the midline of the ventral medulla (raphe magnus), only opioids and not baclofen are antinociceptive whereas in the lateral region (nucleus gigantocellularis) only baclofen and not morphine is antinociceptive (Levy & Proudfit, 1979). In the present study, PAG neurones which were hyperpolarized by opioids were also hyperpolarized by baclofen, but the uniformity of responses to baclofen raises the possibility that other circuits involved in antinociception are also modulated by GABA$_B$ receptors in PAG. Taken together, these findings suggest that systemically administered baclofen and opioids act on partially overlapping antinociceptive systems. This could partly explain potentiation by baclofen of opioid-induced antinociception in animals (Cutting & Jordan, 1975), as well as potentiation or prolongation by baclofen of opiate analgesia in human subjects (Corli *et al.*, 1984; Panerai *et al.*, 1985).

In conclusion, baclofen acting on GABA$_B$ receptors hyperpolarized all 66 PAG neurones tested as a result of increased inwardly rectifying potassium conductance. This inhibitory action is presumably responsible for the antinociceptive effects of baclofen in PAG *in vivo*. The ubiquitous inhibitory actions of baclofen in PAG neurones raises possible questions concerning the validity of models of antinociception which simply postulate that antinociception is mediated by disinhibition of neurones projecting from PAG to the ventral medulla. The profound inhibition of neurones throughout the lateral and ventrolateral PAG by baclofen also suggests that GABA$_B$ receptor activation may play a role in other functions of PAG such as integration of somatic and autonomic components of defence and escape behaviours, and cardio-respiratory functions (see Lovick, 1992; Bandler & Shipley, 1994).

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