Electrophysiological actions of alfentanil: intracellular studies in the rat locus coeruleus neurones

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- 1 The electrophysiological effects of alfentanil on 156 neurones of the rat locus coeruleus were investigated by use of intracellular recordings from the *in vitro* brain slice preparation.
- 2 Bath application of alfentanil (5–100 nM) reversibly decreased the firing rate of all neurones tested in a dose-dependent manner, with an IC_{50} 4.1 nM.
- 3 Based on inhibition of the spontaneous firing rate, alfentanil was 22 times more potent than morphine.
- 4 At 100 nM, alfentanil produced a complete inhibition of firing of all neurones tested ($n = 62$); the inhibition was accompanied by a membrane hyperpolarization 17.0 ± 0.8 mV (range 6.1–30.3 mV, $n = 62$) and a reduction in input resistance $26.4 \pm 1.7\%$ (range 6.5–53%, $n = 51$).
- 5 The effects of alfentanil were antagonized by naloxone, with a dissociation equilibrium constant of 2.7 ± 0.4 nM ($n = 6$).
- 6 The reversal potential for the alfentanil-induced hyperpolarization was -110 ± 2 mV ($n = 9$), which is approximately the potassium equilibrium potential.
- 7 The alfentanil-induced hyperpolarization was blocked by caesium chloride and barium chloride.
- 8 These results indicate that alfentanil binds to μ -opioid receptors on the cell membrane of neurones of the locus coeruleus. This leads to opening of the inward-going rectification potassium channels, resulting in the observed hyperpolarization of the membrane.

Keywords: Alfentanil; locus coeruleus; brain slice; intracellular recording; μ -opioid receptors; potassium channels

Introduction

The 4-anilinopiperidine series of analgesic/anaesthetics are potent, synthetic opioids which are structurally and physico-chemically dissimilar from morphine. Of this series alfentanil, fentanyl and sufentanil are representative compounds (Cookson *et al.*, 1983). In recent years, agents of this novel class have become extremely popular in clinical anaesthesia because of the superior analgesic potency and cardiovascular stability that they provide (Sebel *et al.*, 1982). According to animal studies, the analgesic potency of alfentanil is 30 to 73 times greater than morphine, one fourth as great as fentanyl, and one sixty-sixth as great as sufentanil (Niemegeer & Janssen, 1981; Janssen, 1984). Studies of *in vitro* receptor binding have shown that these 4-anilinopiperidine derivatives possess high opiate receptor affinities, primarily at the μ -receptor site (Leysen *et al.*, 1983). Little is known about the mechanism of action of anilinopiperidine derivatives in the central nervous system.

The hippocampal formation is the most commonly used brain structure for the study of anaesthetic effects, which have been extensively described both *in vivo* and *in vitro* (for a review, see Pocock & Richards, 1991). We have conducted a detailed assessment of the actions of alfentanil on neurones of locus coeruleus (LC) using intracellular recording analysis techniques. The LC brain slice was the subject of the present investigation for three reasons. First, the LC contains the greatest concentration of noradrenergic cell bodies in the central nervous system (CNS), with projections from this small pontine nucleus responsible for more than half the noradrenaline in the brain (Amaral & Sinnamon, 1977). Since the LC has extensive projections to many CNS areas, alterations of the activity of LC neurones would be expected to cause widespread effects in CNS. Second, immunocytochemical and immunofluorescent studies showed that nerve

terminals that contain opioid peptides innervate neurones of the LC (Finley *et al.*, 1981; Léger *et al.*, 1983). Electrophysiological studies also indicate that opioid receptors in the LC are of the μ type (Williams & North, 1984; North, 1986). Third, the LC is the primary origin of descending noradrenergic analgesic fibres (for reviews see Proudfit, 1988; Lipp, 1991; Jones, 1991). Electrophysiological studies have provided strong evidence for a role of LC in modulating analgesia. Lesions of the LC impair the ability of morphine to suppress responses to electrical stimuli of the rat fore paw (Sasa *et al.*, 1977), and analgesia induced by electrical stimulation of LC neurones has been demonstrated in several studies (see Proudfit, 1988 for references). Furthermore, microinjection studies have identified several brain regions as important in mediating opiate analgesia, including the periaqueductal gray, LC, nucleus raphe magnus and nucleus reticularis gigantocellularis (see Bodnar *et al.*, 1988 for references). Bodnar *et al.* (1988) also observed profound analgesia following the microinjection of morphine into these four brain regions and further claimed that morphine analgesia was more potent in the LC than in the periaqueductal gray. Recently, synergistic analgesic interactions between these supraspinal analgesic systems have been reported and nucleus raphe magnus is suggested to form a relay or the final common pathway for opioid-induced supraspinal analgesia (Bodnar *et al.*, 1991; Lipp, 1991; Rossi *et al.*, 1992).

Methods

Preparation and maintenance of slices of locus coeruleus

The methods used to prepare and maintain slices of locus coeruleus of the rat were similar to those previously described (Shefner & Chiu, 1986; Chiu *et al.*, 1990). In brief,

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male Sprague-Dawley rats (120–200 g) were anaesthetized with ether and their brains were rapidly removed. A block of tissue containing the pons was excised and attached to a small Plexiglass stage with cyanoacrylate glue; an agar block, next to the tissue, served to support it during sectioning. The tissue was then submerged in oxygenated artificial cerebrospinal fluid (artificial CSF), maintained at 3–5°C, in the well of a Lancer 1000 vibratome and coronal slices of pons (300–350 μm thick) were cut. A slice containing a cross-section through the caudal end of the locus coeruleus was mounted in the recording chamber and allowed to equilibrate for 1 h. The recording chamber consisted of a Plexiglass bath (0.3 ml) with a nylon net stretched tightly across it. The slice was placed on a square of lens paper on top of the net and an electron microscopy grid, held down by platinum wires, was placed over the locus coeruleus to keep the slice from floating. The slice was completely submerged in artificial CSF of the following composition (mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, NaHCO_3 26.2, MgCl_2 1.3, CaCl_2 2.4, glucose 11.1 (saturated with 95% O_2 /5% CO_2 , pH = 7.4). The temperature of the artificial CSF was 33–34°C and the flow rate was 2.3 ml min^{-1} .

Intracellular recording

The slice was viewed from above through a dissection microscope. In the trans-illuminated slice, the locus coeruleus is seen as a translucent area lying on the lateral aspect of the periventricular gray, below the fourth ventricle. Recording microelectrodes, filled with KCl (2 M) and having d.c. tip resistance 40–70 $\text{M}\Omega$, were used. They were inserted into the locus coeruleus under visual control. Intracellular potentials were recorded by an amplifier with an active bridge circuit, allowing the injection of current through the recording electrode (WPI M707). Current and voltage traces were displayed on a storage oscilloscope (Textronix 5113) and a rectilinear pen recorder (Gould 2400). Input resistance was measured by passing pulses of hyperpolarizing current of varied amplitudes; the resulting alterations of voltage were measured and voltage-current (V/I) curves were constructed. Current pulses were of sufficient duration (250–300 ms) to charge fully the membrane capacitance and to reach a steady-state voltage deflection.

Perfusion of solutions and drugs

A valve system was used to switch solutions, superfusing the preparation, from control artificial CSF to CSF which contained drugs. The period required for test solutions to reach the chamber was known and was in the range 25–35 s. Equilibration to the full concentration of the drug in the chamber took approximately 2.5 min. Drugs were administered for a sufficient period to reach a steady-state response and the effects were observed at 5 min or longer, after the beginning of infusion of the drug.

Drugs

The following drugs were used: alfentanil hydrochloride (Janssen), morphine sulphate (Narcotics Bureau, Taiwan), naloxone hydrochloride, caesium chloride and barium chloride (Sigma). Numerical data are expressed as mean $\pm$ standard error of the mean (s.e.mean).

Results

The present results are based on recordings made from 156 locus coeruleus neurones in total, with stable intracellular impalements. These cells had resting membrane potentials of -50 – -71.7 mV (-60.7 ± 0.4 mV, $n = 156$) and apparent input resistance of 101–362 $\text{M}\Omega$ (161 ± 5 $\text{M}\Omega$, $n = 141$). They fired spontaneously from 0.4 to 3.8 Hz (2.2 ± 0.1 Hz,

$n = 156$), with a regular interspike interval characteristic of neurones of the locus coeruleus in the slice preparation (Williams *et al.*, 1984).

Alfentanil responses

Alfentanil (5–100 nM) reversibly decreased the firing rate of all neurones of the locus coeruleus tested. Although neurones varied in their sensitivity to alfentanil, the inhibition of firing rate, hyperpolarization of the membrane potential and reduction in input resistance depended on concentration (Figure 1). The most sensitive of these effects, which caused a remarkable change even at small alfentanil concentrations, was decrease of the firing rate. At 5 and 10 nM alfentanil, which respectively gave 58% ($n = 14$, including 3 cells in which firing was completely suppressed) and 76% ($n = 29$, including 12 cells in which firing was completely suppressed) inhibition of the neuronal firing rate, little hyperpolarization and small reduction of input resistance were seen. Larger concentrations of alfentanil (30 and 50 nM) caused not only greater inhibition of firing but also hyperpolarization of the membrane potential and decreased input resistance. There was considerable variation of the size of hyperpolarizations and reductions of input resistance induced by the same concentration of alfentanil in different neurones. For instance, the amplitudes of hyperpolarization and reduction of input resistance in response to 30 nM alfentanil were in the range 0–18 mV (9.1 ± 0.7 mV, $n = 47$) and from 0 to 37% ($12.7 \pm 1.4\%$, $n = 40$), respectively. At 100 nM, alfentanil produced complete inhibition of firing of all neurones tested ($n = 62$); the inhibition was associated with a 17 mV hyperpolarization (range 6.1–30.3 mV, $n = 62$) and a 26% reduction in input resistance (range 6.5–53%, $n = 51$). Because locus coeruleus neurones have a pronounced inward rectification, we demonstrated that the decrease of input resistance observed as an effect of alfentanil was not due to membrane rectification induced by the hyperpolarization. The resistance decrease persisted when the membrane potential was manually clamped back to its predrug value during the peak of the effect (e.g. Figure 5b).

Comparison of potencies of alfentanil and morphine

Dose-response curves for alfentanil and morphine for the inhibition of firing rate were compared in 5 neurones of the locus coeruleus. The neurones were perfused individually with one of the two drugs at varied concentrations and then with those of the other. Figure 2 shows the results from one cell, in which alfentanil was 21 times more potent than morphine. A comparison of the IC_{50} between alfentanil and morphine in these 5 neurones is listed in Table 1. On average, alfentanil was 22 times more potent than morphine. In three

Figure 1 Dose-dependent effects of alfentanil on the firing rate ($\square$), membrane potential ($\blacksquare$) and input resistance ($\circ$) of neurones of the locus coeruleus. Mean $\pm$ s.e.mean is shown for the number of neurones indicated.

Figure 2 The dose-response curves for alfentanil (○) and morphine (●) were compared on the inhibition of neuronal firing in the locus coeruleus. The IC_{50} is defined as the concentration which gave 50% inhibition of neuronal firing. The calculated IC_{50} of alfentanil, obtained from this figure, was 2.6 nM; that of morphine was 55.2 nM. In this cell, alfentanil was 21 times more potent than morphine.

Table 1 IC_{50} of morphine and alfentanil for inhibition of spontaneous firing of neurones of the locus coeruleus

Cell no.	IC_{50} of morphine (nM)	IC_{50} of alfentanil (nM)	IC_{50} ratio (Morph/Alf)
1	72.9	2.7	27.2
2	59.2	2.3	25.8
3	55.2	2.6	21.0
4	52.1	2.5	20.9
5	32.2	2.6	12.6
Mean $\pm$ s.e.mean	54.3 $\pm$ 6.6	2.5 $\pm$ 0.1	21.5

The dose-response curve of cell 3 is shown in Figure 2.

other neurones, alfentanil and morphine at greater concentrations were applied to induce stronger hyperpolarization in the LC neurones. These concentration-dependent hyperpolarizations were used to construct dose-response curves. However, it was generally impracticable to construct full concentration-hyperpolarization sigmoidal curves for both alfentanil and morphine during the course of a recording from a single cell. The rank order of potency of alfentanil and morphine was, therefore, determined by comparing their equieffective concentrations that produced a 15-mV hyperpolarization (the half-maximal response caused by 100 μ M normorphine; see Williams & North, 1984). The equieffective concentration of morphine was 25 times greater than that for alfentanil.

Current-voltage relationship before and after perfusion of alfentanil

The current-voltage relationship of the locus coeruleus neurones revealed an anomalous rectification that has been described previously (Andrade & Aghajanian, 1984; Osmanovic & Shefner, 1987; Williams *et al.*, 1988). That the value of the alfentanil reversal potential was so negative made it difficult to reverse alfentanil responses by hyperpolarizing the membrane with d.c. current injection. Instead, the reversal potential for alfentanil-induced hyperpolarization was calculated from the intersection of the control current-voltage curve and the curve obtained in the presence of alfentanil. The relationship between injected current pulses and steady-state membrane potentials is shown in Figure 3c.

Figure 3 Effect of alfentanil on the current-voltage relationship of a neurone in the locus coeruleus. (a) The injected hyperpolarizing current pulses and the generated electrotonic potentials of the neurone, perfused with normal artificial CSF. (b) The current pulses and electrotonic potentials of the same neurone, perfused with alfentanil (100 nM). (c) Current-voltage curves for this neurone, perfused with (○) control artificial CSF and (▲) alfentanil (100 nM). The arrow indicates the reversal potential, which was -115 mV in this neurone.

After alfentanil administration the slope of the current-voltage curve was reduced throughout the voltage range examined; hence the decreased input resistance elicited by alfentanil resulted not simply from anomalous rectification by the cell membrane. The alfentanil reversal potential ranged from -101 to -117 mV; the average was -110 ± 2 mV ($n = 9$).

Effect of naloxone on reversing the hyperpolarization caused by alfentanil

Perfusion of naloxone (100 nM) produced no significant change of membrane potential or input resistance in neurones of the locus coeruleus. However, a small increase in firing rate was occasionally observed. Pretreatment with naloxone (100 nM) and then changing the perfusate to alfentanil (100 nM), mixed with naloxone (100 nM), eliminated the effects of alfentanil on these neurones. Neither firing rate, membrane potential nor input resistance was altered, compared with the pre-alfentanil state ($n = 4$). In another six neurones, the duration of recording was sufficient to construct concentration-response curves for alfentanil, in the absence and presence of naloxone; an example is presented in Figure 4. Naloxone produced a parallel, dose-related shift to the right of the concentration-response curve for alfentanil. To determine the dissociation equilibrium constants (K_D) for naloxone, we calculated the dose-ratio by dividing the concentration of alfentanil, required to produce a given hyperpolarization in the presence of naloxone, by the concentration of alfentanil, required to give an equal hyperpolarization in the absence of naloxone. These dose-ratios were then used to construct the Schild plots, described by Williams & North (1984). These experiments yielded a K_D value of 2.7 ± 0.4 nM ($n = 6$) for the antagonism of alfentanil-induced hyperpolarizations by naloxone.

Figure 4 Antagonism by naloxone of alfentanil-induced hyperpolarization. (a) Shows hyperpolarization induced by superfusion of alfentanil (9900 nM) in the presence of naloxone (300 nM). (b) Dose-response curves for alfentanil in the presence of different concentrations of naloxone: control (●); naloxone 30 nM (○); 100 nM (△) and 300 nM (▲). (c) Schild plot of data shown in (b), at a response level 14 mV. The dissociation equilibrium constant was 2.9 nM in this neurone.

Effect of BaCl₂ and CsCl on blocking hyperpolarization caused by alfentanil

The blocking effects of barium (0.3 mM) and caesium (3 mM) on alfentanil-induced hyperpolarization were tested in 8 and 5 neurones of the locus coeruleus, respectively. Barium depolarized the membrane and increased the amplitude and duration of the action potential. Caesium also increased the peak amplitude, duration and afterhyperpolarization of the neuronal action potential. As shown in Figure 5b, superfusion with alfentanil (100 nM) for 4.5 min produced complete inhibition of spontaneous firing, hyperpolarization of the membrane potential (16.5 mV) and decreased input resistance (25%). After superfusion with caesium chloride (3 mM) for 25 min, or barium chloride (0.3 mM) for 15 min, the same neurone was challenged with alfentanil (100 nM), again in the presence of caesium (Figure 5c) or barium (Figure 5d). During caesium treatment, alfentanil-induced hyperpolarization still occurred, though to a lesser degree (67% reduction). Barium, on the other hand, completely prevented alfentanil-induced hyperpolarization, but inhibition of spontaneous firing remained (a 37% reduction). In eight neurones tested, barium completely blocked the hyperpolarization caused by alfentanil, but a partial suppression of the baseline firing rate was still observed ($48 \pm 10\%$, $n = 8$). In the caesium-treated neurones, caesium reduced but did not eliminate hyperpolarization of alfentanil. On the average, caesium reduced the alfentanil-induced hyperpolarization by 44% of these five cells.

Discussion

Our investigation provides evidence that alfentanil is able to inhibit the spontaneous firing rate, cause hyperpolarization of the membrane potential and reduce the input resistance of neurones of the locus coeruleus. These findings are consistent with those of previous studies that indicate that many anaesthetic agents reduce the neuronal excitability of central neurones by increasing membrane conductance and producing hyperpolarization (Nicoll & Madison, 1982; Berg-Johnsen & Langmoen, 1987; O'Beirne *et al.*, 1987). The functional significance of this hyperpolarization would be to increase the threshold of impulse initiation and thus to decrease the probability that a cell discharges in response to a given excitatory synaptic input. By use of extracellular single neurone recording, it has been demonstrated that alfentanil

inhibited both the spontaneous activity of medullary respiratory neurones in rats (Hewson & Bradley, 1982) and the noxiously evoked activity of cells in the nucleus reticularis gigantocellularis in cats (Yuge *et al.*, 1985). The present results on the spontaneous neuronal activity are compatible with the extracellular findings. We also found that the depressant effects of alfentanil on neurones of the locus coeruleus were more potent than those of morphine. Comparison of the ability of alfentanil and morphine to inhibit neuronal firing in the locus coeruleus showed that alfentanil was 22 times as potent as morphine. Although in some studies no evidence was found of alteration of membrane potential with anaesthetic agent treatment (Weakly, 1969; Zorychta & Capek, 1978; Sawada & Yamamoto, 1985; Richards & Strupinski, 1986), the present results that alfentanil is 25 times as potent as morphine in causing hyperpolarization confirm previous observations that there is a clear correlation between the anaesthetic (or analgesic) potency and the hyperpolarization potency (Nicoll & Madison, 1982, see Figure 1).

The observation that naloxone was able to counteract the inhibitory effect of alfentanil on neurones of the locus coeruleus is consistent with previous reports, which demonstrated that naloxone competitively reversed the effects of opioid substances ([D-Ala², N-methyl-Phe⁴, Gly⁵-ol]enkephalin), with a dissociation equilibrium constant in the range 1.5–2 nM (Williams & North, 1984; McFadzean *et al.*, 1987). Our work showed that naloxone antagonized the actions of alfentanil, with a dissociation equilibrium constant about 2.7 nM, which is within the range used to define the μ -opiate receptor (0.5–3 nM, North, 1986) and appears to be much smaller than the antagonistic K_D values for naloxone on δ - and κ -receptors (30 and 15 nM, respectively; North, 1986, Table 1). North *et al.* (1987) also demonstrated that the μ -agonist, and not the δ -agonist, caused hyperpolarization of neurones of the locus coeruleus. Extrapolating from these results, we suggest that hyperpolarization caused by alfentanil was also mediated by μ -opiate receptors (Leyssen *et al.*, 1983).

Hyperpolarization of neurones of the locus coeruleus, caused by alfentanil, appears to be due to the opening of potassium channels, that display anomalous rectification, for the following reasons: (1) alfentanil enhanced anomalous rectification; (2) caesium and barium suppressed alfentanil-induced hyperpolarization, and (3) the reversal potential of the hyperpolarization was about -110 mV, approximately the potassium equilibrium potential (Williams *et al.*, 1988).

This evidence is consistent with previous studies that indicated that the opioid-induced potassium conductances in neurones of the submucous plexus (North *et al.*, 1987), the arcuate nucleus (Loose & Kelly, 1990) and locus coeruleus (North *et al.*, 1987; Williams *et al.*, 1988) are characterized by inward rectification. Caesium chloride, however, can only partially suppress alfentanil-induced hyperpolarization in the neurones tested (see also North & Williams, 1985; Chiu *et al.*, 1990). In contrast, hyperpolarization induced by alfentanil was completely suppressed by barium in the neurones tested. These data indicate that barium was more effective than caesium in suppressing alfentanil-induced hyperpolarization. Extracellular barium and caesium block inward-

rectifying potassium channels in the LC (Osmanovic & Shefner, 1987; Williams *et al.*, 1988) but the behaviour of caesium blocking differs from that of barium blocking. Externally applied caesium appeared to cause only a voltage-dependent block of the inward potassium current through the inward rectifier but had no effect on the outward potassium current induced by opioids (North *et al.*, 1987) or somatostatin (Mihara *et al.*, 1987). External barium, in contrast, can block both inward and outward potassium currents (see Constanti & Galvan, 1983 for references). Furthermore, barium carries charge through calcium channels more effectively than calcium itself in the LC (Williams *et al.*, 1984) and in several other kinds of excitable membrane (see Schwandt & Crill,

Figure 5 Effects of caesium and barium on alfentanil-induced hyperpolarization in a neurone in the locus coeruleus. (a) Shows the injected current with amplitude 0.065 nA, duration 300 ms and frequency 0.3 Hz. (b) Superfusion with alfentanil (100 nM) caused complete inhibition of the firing rate, hyperpolarization of the membrane potential (16.5 mV) and a decrease in input resistance (25%). During the steady state of hyperpolarization, a d.c. depolarizing current was injected to return the membrane potential of this cell to its predrug level (see current trace in a). When this was done, the input resistance was still decreased, as compared with the predrug state. (c) In the presence of caesium (3 mM), alfentanil-induced hyperpolarization still occurred, though to a lesser degree (a 67% reduction). (d) In the presence of barium (0.3 mM), alfentanil (100 nM) produced no hyperpolarization. However, inhibition of spontaneous firing still occurred, although it was not complete (37% inhibition).

1980, for references), which in turn would generate a depolarizing current. In combination, these effects of barium would contribute to its actions in opposing the alfentanil-induced hyperpolarization.

The mechanisms by which the LC involved in the mediation of opioid analgesia are complicated as there appears to be interaction between the supraspinal systems. A review of the literature indicates that participation of the LC in modulating morphine-induced antinociception is mediated indirectly through the nucleus raphe magnus (Lipp, 1991). Neurones in two classes are identified in the nucleus raphe magnus, termed off- and on-cells (Fields *et al.*, 1988; see also Pan *et al.*, 1990). Several lines of evidence indicate that on-cells function primarily as intrinsic GABAergic interneurons that inhibit off-cells and that disinhibition (activation) of off-cells results in antinociception (Pan *et al.*, 1990; Fields *et al.*, 1991; Heinricher *et al.*, 1992). Pharmacological investigations have shown that on-cells but not off-cells were consistently and selectively excited by noradrenaline, and this facilitatory effect was reversed by the α_1 -antagonist, prazosin

(Heinricher *et al.*, 1988). Since the nucleus raphe magnus receives noradrenergic innervation from the LC (Chu & Bloom, 1974; Amaral & Sinnamon, 1977; Sakai *et al.*, 1979; Westlund & Coulter, 1980; Dong & Shen, 1986), then direct inhibition of LC neurones by opioid substances (e.g. alfentanil) results in decreased release of noradrenaline within the nucleus raphe magnus. Consequently, lifting of the tonic excitatory influence of noradrenaline on the on-cells disrupts GABA inhibition of the off-cells. This disinhibition allows the descending inhibitory influence of off-cells on the spinal pain-transmission to be facilitated and could be the possible mechanism by which the LC mediates opioid (alfentanil) supraspinal analgesia (Lipp, 1991; Field *et al.*, 1991; Heinricher *et al.*, 1992; Heinricher & Tortorici, 1992).

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