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Opiate-Receptor Interactions on Single Locus Coeruleus Neurones

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SUMMARY

Intracellular recordings were made from neurones of the rat locus coeruleus (LC) which were located in a slice of pons superfused *in vitro*. Opioid agonists and antagonists were applied by adding them to the superfusing solution; normorphine and enkephalin analogues were also applied by ejecting a few nanoliters of a solution which contained the drugs from a pipette situated above the tissue slice. Opioid agonists hyperpolarized LC neurones. This has been shown previously to result from an increase in the membrane potassium conductance. The lowest concentration of normorphine which was effective was 30 nM, the EC_{50} was 1 μM, and the maximum effect was observed with 30 μM. The irreversible antagonist β-funaltrexamine (β-FNA) was used to estimate the dissociation equilibrium constants; these ranged from 9–16 μM for normorphine and [Met^5]enkephalin and was about 2 μM for [D-Ala^2,D-Leu^5]enkephalin. β-FNA also blocked the hyperpolarization caused by [D-Ala^2,D-Leu^5]enkephalin, ethylketacyclazocine, and [D-Ser^2,D-Leu^5] enkephalin-Thr. Naloxone reversibly antagonized the hyperpolarizations caused by normorphine and [D-Ala^2,D-Leu^5]enkephalin, with a dissociation equilibrium constant of 2 nM. It is suggested that the opioid hyperpolarization of LC neurones is mediated by a receptor having a high affinity for naloxone, previously termed a μ-receptor. The affinity of this receptor for normorphine appears to be 3 to 4 orders or magnitude lower than its affinity for naloxone.

INTRODUCTION

Interactions between drugs and receptors are often studied indirectly by observing tissue responses (1–3). These techniques have been of limited value for centrally acting drugs because of the difficulties in measuring a response closely linked in space and time to the presence of the drug at the receptor. In the case of opiates, useful responses have been found in peripheral tissues where muscle contraction or transmitter release can be measured (4, 5). Information about central nervous system opiate receptors has come largely from ligand-binding experiments on homogenized cells (6–8), but there is little direct evidence that binding sites identified by tritiated agonist ligands are causally involved in mediating opiate actions on nerve cell excitability.

We have found recently that opiates hyperpolarize certain central neurones by increasing their membrane potassium conductance (9, 10). Such a hyperpolarization seems likely to underlie the inhibition of neuronal firing which is a widespread feature of opiate action (11, 12). In the present study, we have used the hyperpolarization of single neurones of the rat LC[1] as a tissue response in order to obtain information about the opiate-receptor interaction in the central nervous system. We sought to address two principal questions. First, do the opiate receptors on intact cells have affinities for antagonists similar to those determined in ligand-binding experiments, and would this allow us to ascribe the receptors on LC neurones to one of the currently accepted classes of opiate receptor? Second, what is the affinity of the opiate agonist normorphine for the receptors on LC neurones through which it causes a membrane hyperpolarization and inhibition of cell firing? A preliminary account of the work has been published (13).

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[1]The abbreviations used are: LC, locus coeruleus; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DSLET, [D-Ser^2,Leu^5]enkephalin-Thr; FK 33824, [D-Ala^2,NMePhe^4,Met(O)^5]enkephalin-ol; β-FNA, β-funaltrexamine; EKC, ethylketazocine methane sulfonate; nS, nanosiemen

MATERIALS AND METHODS

Intracellular recordings were made from rat LC neurones by techniques previously described (9, 14–16). Briefly, 300-μm thick transverse sections of pons were completely submerged in a heated (37°), flowing (1–2 ml/min) solution of the following millimolar composition: NaCl, 126; KCl, 2.5; $CaCl_2$, 2.5; NaH_2PO_4, 1.2, $MgCl_2$), 1.2, $NaHCO_3$, 25; glucose, 11; this was gassed with 95% O_2/5% CO_2. In some experiments membrane current was measured by single electrode voltage-clamp technique (Axoclamp II), the details of which have been previously described (16). The following-drugs were applied by changing this superfusing solution to one which differed only in its content of the drug: normorphine sulfamate (Dr. E. May, National Institute of Drug Abuse), meptazinol (Wyeth), [Met^5]enkephalin, DADLE, FK 33824, DSLET, and dynorphin (all from Peninsula), EKC (Dr. Suzanne Zukin, Albert Einstein College of Medicine), cyclazocine (Dr. C. R. Schuster, University of Chicago), *trans*-(±)-3,4-dichloro-*N*-methyl-[2-(1-pyrrol-

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idinyl)cyclohexyl] benzeneacetamide methane sulfonate (U50488H, Upjohn), naloxone hydrochloride (Endo), β-FNA (the 6β-fumaramate methyl ester derivative of naltrexamine, Dr. P. Portoghese, University of Minnesota), phentolamine hydrochloride (Ciba), *N,N*-bisallyl-Tyr-Gly-Gly-ψ-(CH_2S)-Phe-Leu-OH (ICI 154129), *N,N*-bisallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174864), yohimbine hydrochloride (Sigma), and noradrenaline bitartrate (Sigma).

Normorphine, FK 33824, [Met^5]enkephalin, DADLE, and noradrenaline (Sigma) wee also applied by pressure. An aliquot of superfusing solution containing the drug (100 μM) was introduced into a micropipette. The pipette tip (5–15-μm diameter) was positioned above the slice surface over the LC, but beneath the surface of the superfusing solution. Drugs were ejected by applying brief (10–50 msec) pulses of pressure (50–150 kPa) to the pipette (Picospritzer II, General Valve Corporation). Increasing amounts of drug were applied by increasing the number of such pulses applied to the pipette at a frequency of 10 Hz. [^{3}H]Noradrenaline release from such pipettes is linearly related to the number of pulses applied (14).

RESULTS

Agonists. Superfusion of various opioids inhibited the spontaneous firing and hyperpolarized LC neurones. We have previously shown that this hyperpolarization results from an increase in the membrane potassium conductance (9). Examples of the opioid hyperpolarizations recorded in a single LC neurone are shown in Fig. 1. In this experiment, [Met^5]enkephalin was used as the agonist but DADLE and normorphine had similar though somewhat longer lasting effects. Opioid hyperpolarizations recorded from single neurones continued without sign of "desensitization" throughout periods of superfusion as long as 8 hr. Repeated application of normorphine caused reproducible hyperpolarizations of LC neurones for up to 15 hr.

Figure 1 shows that the hyperpolarization caused by [Met^5]enkephalin in a given neurone was dependent on the concentration applied. Figure 2A summarizes the effects of normorphine superfusion on the membrane potential of many neurones. It is apparent that there was only small variability in the sensitivity of individual neurones; all LC neurones were hyperpolarized. The

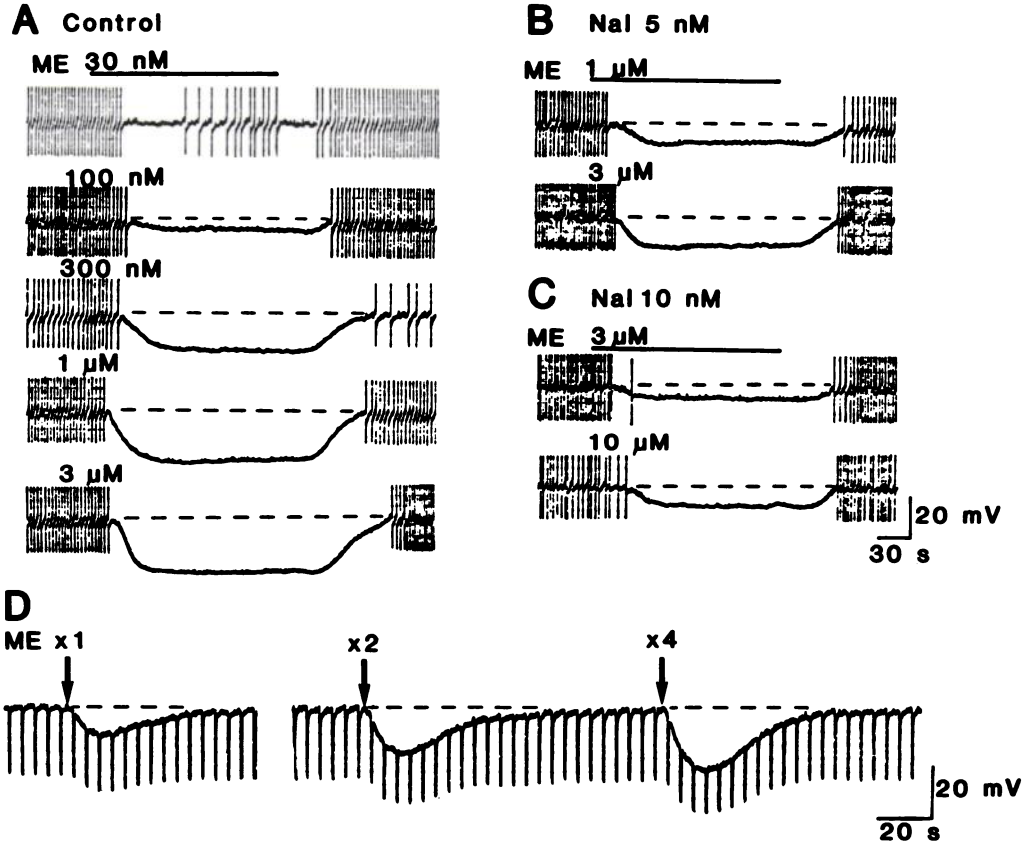

FIG. 1. *Hyperpolarization of single LC neurone by [Met^5]enkephalin*

Intracellular recordings from a spontaneously firing LC neurone. The full amplitude of the action potentials is not reproduced in this or other chart records. In all figures, the superfusion solution was changed to one containing agonists at the given concentration during the period indicated by the bar above the trace. A, superfusion of [Met^5]enkephalin (*ME*) resulted in hyperpolarizations which were concentration dependent. Spontaneous firing was decreased with little or no potential change by 30 nM, and the maximum hyperpolarization (26 mV) was produced by 1 μM. B, effects of [Met^5]enkephalin on the same cell after superfusion with naloxone (5 nM) for 20 min. C, the same cell after superfusion with naloxone (10 nM) for 20 min. The naloxone antagonism of the action of [Met^5]enkephalin was surmountable. - - - indicates −55mV. D, pressure ejection of [Met^5]enkephalin resulted in dose-related hyperpolarizations. Pressure pulses (10 msec/105 kPa) were applied at the arrows (number of pulses indicated). The amplitude of the hyperpolarization peaked 15 sec after the pressure application and passed off within 1 min. Downward deflections are electrotonic potentials resulting from constant current pulses being passed through the recording electrode across the membrane. Constant hyperpolarizing current was passed to prevent spontaneous action potentials and hold the resting membrane potential at −60 mV.

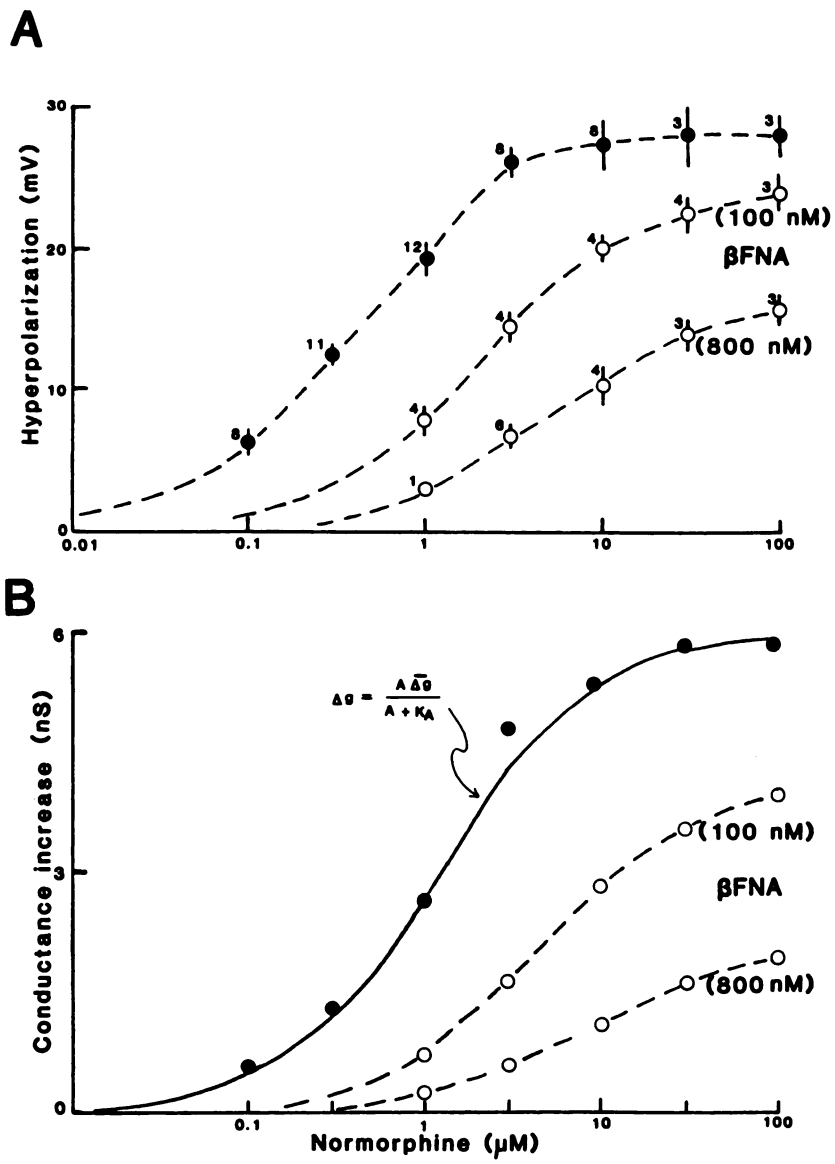

FIG. 2. *Dose-response curves of LC neurones to normorphine before and after treatment with β-FNA*

The hyperpolarization caused by various concentrations of normorphine are shown for several neurones in control slices and in slices previously treated with β-FNA. A, control (●). The amplitude of the hyperpolarization was plotted as a function of the concentration of normorphine applied by superfusion. In all cells, a constant hyperpolarizing current was passed which held the membrane potential at −60 mV prior to normorphine application; this was to prevent spontaneous action potentials. No less than three concentrations of normorphine were tested on each neurone. [In each experiment, naloxone (1 or 3 μM) was applied following application of the highest concentration of normorphine. This blocked completely the hyperpolarization produced by any concentration of normorphine tested.] ○, results of similar experiments on slices which had first been treated with β-FNA (100 or 800 nM) for 30 min and washed for 30 min. B, the dose-response curves from the data shown in A after converting the normorphine hyperpolarization to conductance increase (see text). ●, control. —, rectangular hyperbola (see text). ○, mean values after β-FNA (100 and 800 nM). - - -, fitted by eye.

lowest concentrations of normorphine and [Met^5]enkephalin tested (30–100 nM) slowed the rate of discharge of the spontaneous action potentials. This spontaneous firing could be prevented by passing sufficient current through the recording electrode (usually 20–50 pA); in these circumstances, the lowest opioid concentrations caused a hyperpolarization of 2–5 mV. We have previously found that the spontaneous firing of LC neurones results from a persistent inward calcium current (16). The inhibition of firing with the lowest concentrations of the opioid agonists presumably results from the activation of an opposing outward current. Firing will be inhibited when this outward current balances the persistent inward current; higher opioid concentrations cause a further increase in outward current and this hyperpolarizes the cell (see below).

The maximum hyperpolarization observed was about 30 mV (Fig. 2A). It might be thought that this results from the reduced ion potassium ion driving force as the membrane potential approaches the potassium equilibrium potential (E_K). However, in these neurones, the control membrane potential was about −55 mV, and E_K was about −110 mV (9, 15). This implies that the opioid-elicited conductance reaches a maximum value. The

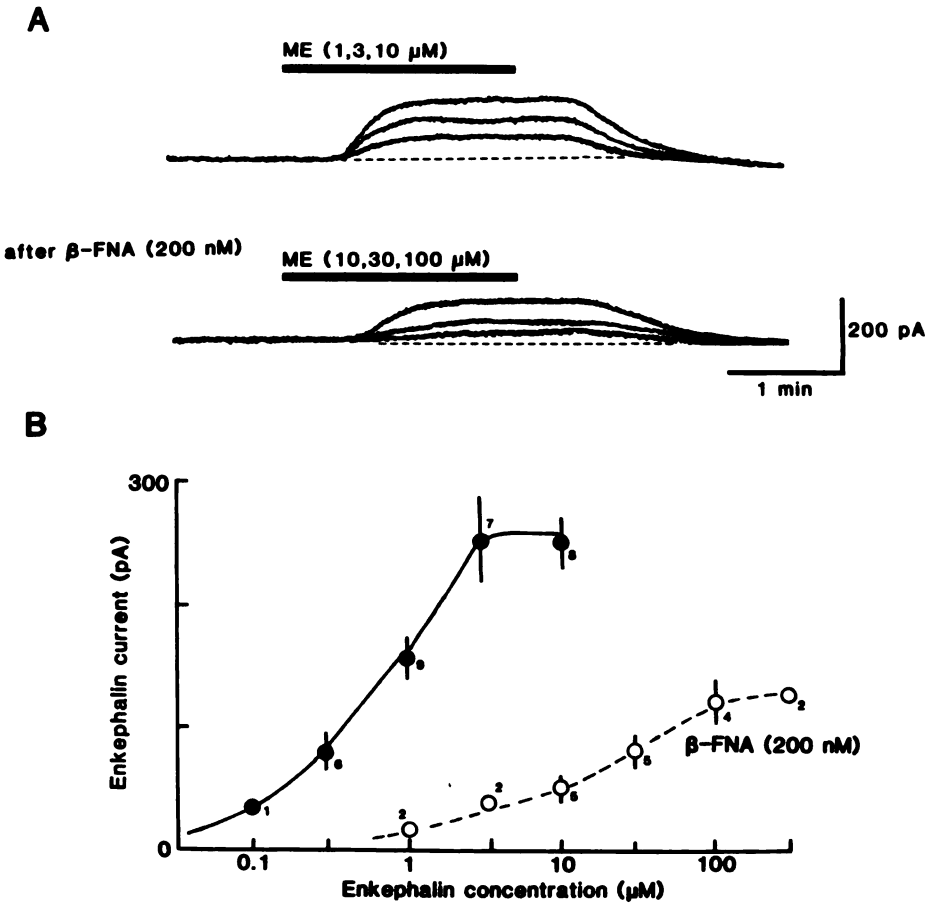

FIG. 3. *Irreversible reduction by β-FNA of [Met⁵]enkephalin-induced outward current*

A, top trace shows three superimposed records of membrane current during recorded from one neurone. The solid bar indicates the period during which the superfusing solution contained [Met⁵]enkephalin in the concentrations indicated. The bottom trace shows the effect of reapplying the [Met⁵]enkephalin to the same cell after a 20-min treatment with β-FNA (200 nM). Holding potential was −60 mV. B, concentration-response curves for [Met⁵]enkephalin before and after β-FNA, derived from the type of experiment illustrated in A. Bars represent standard error of mean for the number of observations indicated. The maximum outward current was substantially reduced by β-FNA.

opioid conductance increase could be calculated from the hyperpolarization or from direct measurement of membrane current. In the case of normorphine, the conductance change calculated from the hyperpolarization is shown in Fig. 2B, assuming a maximum conductance of 6 nS. The concentration of normorphine causing half-maximal conductance increase was 1.2 μM. Figure 3 shows the outward current measured directly as a function of the [Met⁵]enkephalin concentration. The peak current (at 10 μM) was 247 ± 22 pA (mean ± standard error, $n = 8$) and the [Met⁵]enkephalin EC_{50} was 650 nM. Similar experiments with DADLE gave a peak current of 220 ± 41 pA ($n = 4$), with an EC_{50} of 150 nM. The results indicate that there is a limit to the conductance increase which opioids can produce, and this is approximately 5 nS.

The rank order of potency of various agonists was determined by measuring the lowest agonist concentration which produced a 5-mV hyperpolarization. This was: FK 33824, 10 nM; DADLE, 30 nM; normorphine, 100 nM; ethylketazocine, 300 nM; DSLET, 1 μM. Drugs which had no effect on membrane potential at the concentrations indicated were: naloxone (1 μM), cyclazocine (3 μM), β-FNA (800 nM), U50488H (10 μM), meptazinol (50 μM) and dynorphin (300 nM).

Pressure ejection of DADLE, [Met⁵]enkephalin, and normorphine produced approximately equivalent hyperpolarizations for the same ejection pressures and times. An example is shown in Fig. 1D. Similar effects were produced by shorter pressure pulses (5–10 msec) applied to pipettes containing FK 33824, and the hyperpolarization induced by FK 33824 lasted two to three times as long as those caused by DADLE, [Met⁵]enkephalin, or normorphine. Increasing the number of pressure pulses applied to the pipette increased the amplitude and duration of the hyperpolarization (Fig. 1D).

The affinity of the opioid agonists for the receptors on LC neurones will be equal to their EC_{50} values only if occupancy and response are directly proportional and there are no spare receptors. The affinities of normorphine, [Met⁵]enkephalin, and DADLE were estimated by the method of partial irreversible antagonism (17), using β-FNA as an irreversible antagonist. Figures 2 and 3 show the hyperpolarizations and outward currents caused by various concentrations of normorphine and [Met⁵]enkephalin before and after β-FNA treatment. A

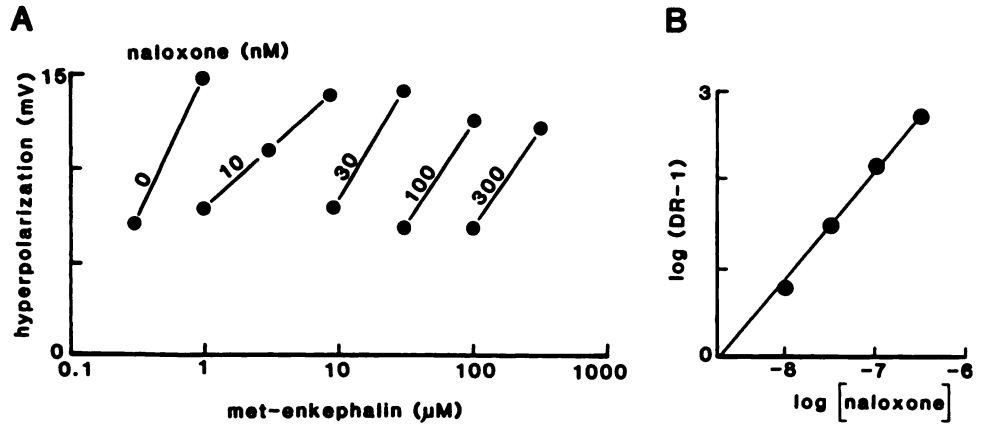

FIG. 4. *Antagonism by naloxone of [Met⁵]enkephalin hyperpolarization*

A, concentration-response curves for [Met⁵]enkephalin for the hyperpolarization of a single LC neurone. Hyperpolarizations were measured from the type of experiment illustrated in Fig. 1A. The numbers beside each trace indicate the concentration of naloxone present. B, Schild plot constructed from the data shown in A.

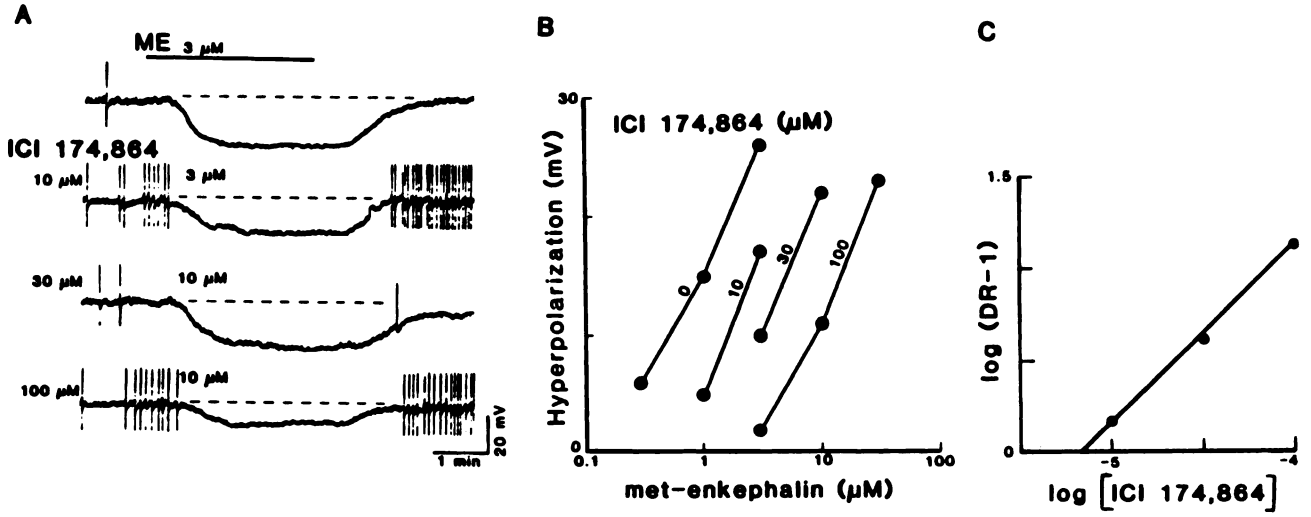

FIG. 5. *Antagonism by ICI 174864 of [Met⁵]enkephalin hyperpolarization*

All parts are derived from recordings from a single neurone. A, intracellular recordings of membrane potential to illustrate the hyperpolarizations induced by [Met⁵]enkephalin superfusion, either alone or in the presence of increasing concentrations of ICI 174864. The period of application of [Met⁵]enkephalin was the same in each trace, and is indicated by the solid bar above the records. B, concentration-response curves showing the effects of all 10 concentrations of [Met⁵]enkephalin applied to this cell. The numbers beside the traces indicate the concentrations of ICI 174864 present. C, Schild plots derived from the data shown in B, at a response level of 16 mV. The line is straight ($r = 0.99$, $p < 0.05$) and has a slope of 0.94. The pA_2 was 5.17.

plot was made of the reciprocals of the concentrations of the agonists giving equal responses before and after treatment with β-FNA, and from the slope and intercept of the line fitting the plotted points, an estimate was made of the proportion of receptors blocked by β-FNA (z) and the dissociation equilibrium constant (K_D) for the agonists (for details of underlying theory, see refs. 17 and 18). In the case of normorphine, these estimates were: with 100 nM β-FNA, $z = 0.85$ and $K_D = 14$ μM; with 800 nM β-FNA, $z = 0.95$ and $K_D = 9$ μM. These results imply that the dissociation equilibrium constant for normorphine is approximately 12 μM, or 10 times the concentration at which it produces half-maximal response. In other words, in our experimental conditions, half-maximal effects are produced at about 5% receptor occupancy. A concentration of normorphine which completely stops cell firing (100 nM) would be expected to occupy about 1% of these receptors. The affinity constants for [Met⁵]enkephalin and DADLE estimated by the same technique (using 200 nM β-FNA) were 16 and 2.2 μM, respectively.

Antagonists. Naloxone prevented the opioid hyperpolarizations when it was applied prior to the agonist, and cut short the hyperpolarization when it was added to the agonist-containing solution. The hyperpolarization caused by superfusion of 1 μM normorphine was completely reversed by 10 nM naloxone. Dose-response curves for normorphine, [Met⁵]enkephalin, and DADLE were constructed by increasing the number of pulses applied to the pressure pipette. Superfusion with naloxone shifted these dose-response curves to the right. The "dose ratio" was calculated by dividing the number of pressure pulses required to produce a given hyperpolarization in the presence of naloxone by the number of

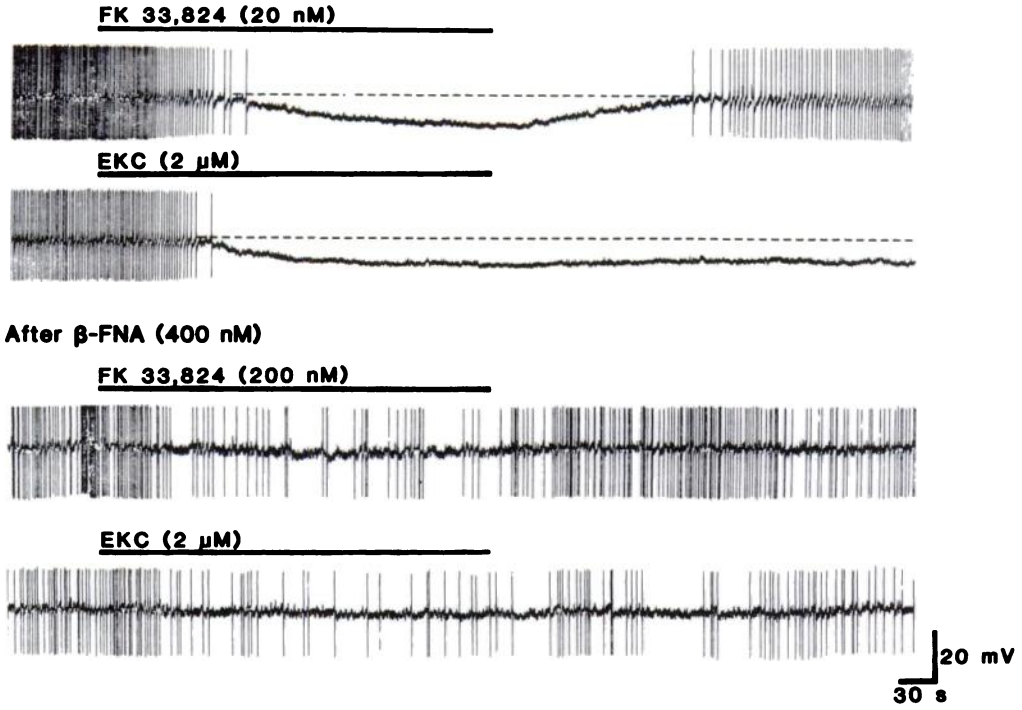

FIG. 6. *β-FNA antagonizes hyperpolarizations caused by FK 33824 and EKC*

Top panel shows hyperpolarizations caused by superfusion with FK 33824 (20 nM) and EKC (2 μM). The EKC hyperpolarization reversed after several minutes (not shown in this trace). Bottom panels show effects of superfusion with FK 33824 (200 nM) or EKC (2 μM) 20 and 40 min after a 20-min exposure to β-FNA (400 nM).

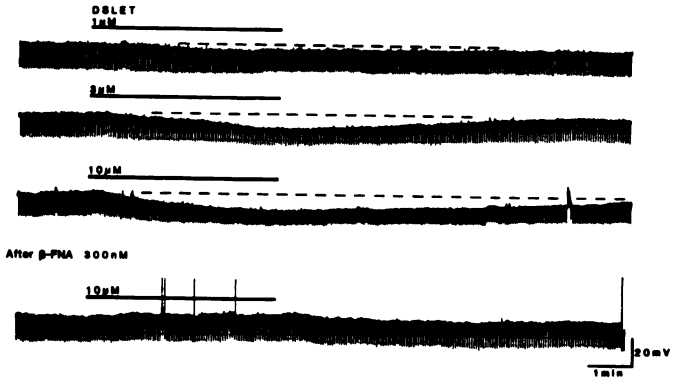

FIG. 7. *β-FNA antagonizes hyperpolarization caused by DSLET*

Concentration-dependent hyperpolarization produced by DSLET. A small constant hyperpolarizing current was applied throughout this experiment to prevent spontaneous action potentials. Downward deflections are electrotonic potentials resulting from fixed amplitude current pulses passed through the recording electrode. Top three recordings show a concentration-dependent hyperpolarization produced by superfusion with DSLET (1, 3, and 10 μM). Bottom trace, following a 20-min superfusion with β-FNA (200 nM). The hyperpolarization produced by superfusion of DSLET (10 μM) was completely antagonized by β-FNA. - - -, −60 mV. We are grateful to Terry Egan for performing the experiment from which this illustration was made.

pulses required to give an equal hyperpolarization in the absence of naloxone (see Fig. 4 of ref. 9). Schild plots (2, 19) were constructed using these dose ratios, and the pA_2 values were determined for naloxone as an antagonist of DADLE and normorphine. These values [8.6 ± 0.2 (6) and 8.5 ± 0.2 (3)] were not different from each other (values are means ± standard error of mean for number of observations indicated). ICI 154129 was also used as an antagonist; the estimates of the pA_2 for ICI 154129 were the same whether DADLE or normorphine was used as agonist [5.2 ± 0.1 (4) and 5.2 ± 0.1 (2), respectively].

The agonist concentrations are not known during experiments with pressure application. Errors in the calculation of dose ratios may therefore contribute to the finding that the Schild plot sometimes had a slope which was different from unity. For all experiments, the mean slope was 1.06 ± 0.12 (n = 15); the slope was not constrained to unity when pA_2 values were calculated in individual experiments. Another source of error in the estimates arises from the difficulty in obtaining dose ratios greater than 16 or 32; in experiments with superfusion of agonist dose ratios of several hundred could be obtained (see below). Despite these reservations about the actual values, the results indicate that both DADLE and normorphine hyperpolarize LC neurones by acting at a similar receptor; this receptor appears to bind naloxone and ICI 154129 with equilibrium dissociation constants of approximately 3 nM and 6 μM.

In four experiments, concentration-response curves were constructed for the agonist [Met^5]enkephalin applied by superfusion, in the presence of at least three different concentrations of naloxone (Figs. 1 and 5) or the more selective δ-antagonist ICI 174864 (20). These experiments yielded straight Schild plots (Figs. 4 and 5); they provided estimates for the naloxone K_D of 2.4 and 0.5 nM, and for the ICI 174864 K_D of 6.8 and 5.2 μM. In four other experiments, the shifts in the concentration-response curves caused by only two concentrations of

naloxone were measured. The value of the naloxone K_D from all six cells was 2.0 ± 0.4 nM (mean ± standard error). Such experiments were possible while recording from a single neurone when [Met^5]enkephalin was used as the agonist; however, the hyperpolarizations caused by superfusion of high normorphine concentrations reversed so slowly (20–60 min) that this approach was not feasible.

Experiments using the irreversible antagonist β-FNA to determine normorphine affinity were described above. β-FNA was equally effective in blocking the hyperpolarizations evoked by normorphine, DADLE, FK 33824, DSLET, and EKC (Figs. 6 and 7). This antagonism was irreversible for up to 6 hr after application of concentrations greater than 200 nM. The blockade caused by 100 nM β-FNA was found to reverse partially after 2 hr of washing. Hyperpolarizations caused by pressure application of noradrenaline, which are also due to an increase in membrane potassium conductance (15), were not changed by β-FNA (800 nM). Conversely, phentolamine (10 μM) or yohimbine (3 μM) blocked noradrenaline-induced hyperpolarizations but did not alter the hyperpolarization produced by any of the opiate agonists.

DISCUSSION

The affinity of agonists. One finding of the present study is that relatively high concentrations of opioid agonists are required to hyperpolarize LC neurones. In our experimental conditions, the normorphine EC_{50} was 1.2 μM. One might argue that the EC_{50} for the hyperpolarization or conductance increase is an inappropriate measure as far as the effects of systemically administered morphine are concerned. Ten to 30 times lower concentrations than the EC_{50} for hyperpolarization stopped spontaneous firing, and once the cell has stopped firing, further hyperpolarization is essentially irrelevant. The concentration of 30 nM which significantly slowed cell firing might be expected to be achieved with a usual therapeutic dose of morphine. For example, Dahlstrom and Paalzow (36) found that an intravenous injection of 2.5 mg/kg morphine in the rat resulted in a medulla-pons morphine level which peaked at 300 nM, remained above 100 nM for 1 hr, and above 30 nM for 2 hr. The same injection of morphine increased the threshold electrical stimulus to the tail which evoked vocalization, their measure of pain relief, and this effect lasted for about 1 hr.

Our estimates of normorphine K_D are subject to certain errors. In particular, it was not possible to obtain a maximum response to normorphine in the presence of β-FNA (Fig. 2). The complete dose-response curves before and after β-FNA could not be completed during recording from a single neurone. On the other hand, the variability in normorphine effect from cell to cell was quite small (Fig. 2) and two separate series of experiments with different concentrations of β-FNA provided approximately the same estimate of the normorphine K_D (9 and 14 μM). This implies that, under our experimental conditions, normorphine acts on LC neurones with a substantial fraction of spare receptors. Similar conclusions were made from studies on the guinea pig ileum with chlornaltrexamine as irreversible antagonist. Porreca and Burks (5) found a K_D for normorphine of 1.6 μM and an EC_{50} of 253 nM.

The estimate of the K_D of [Met^5]enkephalin may be more accurate than that of normorphine for two reasons. First, membrane currents were measured directly and maximum responses could be obtained. Second, the dose-response curves before and after β-FNA could often be completed on the same cell. For both [Met^5]enkephalin and DADLE, the K_D exceeded the EC_{50}. Chavkin and Goldstein (21) found that [Leu^5]enkephalin had a K_D of about 50 μM and an EC_{50} of 200 nM in the guinea pig ileum. The proportion of spare receptors will be expected to vary with the agonist used and with the experimental conditions (3). In our conditions, all three agonists tested appear to act with a substantial proportion of spare receptors. Hill plots constructed from the dose-response data after elimination of spare receptors (after treatment with β-FNA) for individual neurones had slopes close to unity (0.96 ± 0.08, mean ± standard error, $n = 8$) and gave the same values for the K_D as the double reciprocal plots.

The affinity of naloxone. The estimates of naloxone affinity were approximately 3 nM when the agonists normorphine and DADLE were applied by the pressure ejection technique and about 2 nM when [Met^5]enkephalin was applied by superfusion. The main source of error attaching to the estimates using the pressure application arises from two questions. Is the antagonism under equilibrium conditions? Can the ratio of agonist concentrations before and after naloxone be estimated from the ratio of the number of pressure pulses used to eject the agonist? So far as the release of agonist from the pipette is concerned, it has been found that the ejection of [^{3}H]noradrenaline from similar pipettes is linearly related to the number of pressure pulses applied (14); typical pulses in that study ejected 0.1–1 pmol of drug (10 nl of a solution containing 100 μM noradrenaline). One may assume that the agonists in the present study are released from a point source about 300 μm from the impaled neurone (this would correspond to a neurone situated 150 μm within the slice and the tip of the pipette about 150 μm above the slice surface). The time course of the concentration at the cell surface can then be calculated from the diffusion equation (21). Taking a value of 10^{-5} cm^2/sec for the diffusion coefficient (D), we found that the concentration at the neurone reaches its peak at 10–20 sec after the ejection pulse; this compares well with the experimental observations (Fig. 1D). Furthermore, the peak concentrations calculated from the diffusion equation are 1 μM for ejection of 0.1 pmol, 3 μM for 0.3 pmol, 9 μM for 1 pmol, and 27 μM for 3 pmol. From the dose-response curve of Fig. 2A, it can be seen that these concentrations (when applied by superfusion) would be expected to produce hyperpolarizations similar to the peak amplitudes actually observed with pressure application. There are, of course, many reasons to question the assumption of free diffusion in the slice preparation, but these will largely affect the choice of value for D (see ref. 23) The main point of the present discussion is that pressure application of small volumes in short time pe-

riods might be expected to cause peak concentrations at a distant (hundreds of micrometers) site which are proportional to the number of moles applied.

The estimates of the naloxone K_D using superfusion of both agonist and antagonist gave values which were not significantly different from the studies with pressure application. This suggests both that the underlying assumptions in the pressure application technique are appropriate and that a single class of receptors is occupied by [Met^5]enkephalin, DADLE, and normorphine.

The present estimates of naloxone K_D correspond to those determined in the guinea pig ileum (4, 24), implying that the receptor type involved is similar. In the guinea pig ileum, the value for the naloxone K_D (2.8 and 2.6 nM against dihydromorphine and DADLE; ref. 24) agreed well with the dissociation equilibrium constant for naloxone as a displacer of [^{3}H]dihydromorphine (24). Unfortunately, there is no experimental value available for the naloxone K_D determined by ligand binding to the neurones of the LC, but naloxone displaces iodinated FK 33824 from membranes of the whole rat brain with an IC_{50} of about 1 nM (25).

The type of opioid receptor. The naloxone K_D determined in the present study is similar to that found in the guinea pig ileum (4, 24), and close to its dissociation equilibrium constant at binding sites in brain homogenates described as μ-receptors (8, 25). The same value was found whether normorphine or DADLE was used as agonist, indicating that DADLE hyperpolarized LC neurones by occupying a μ-receptor. The experiments with ICI 154129 agree with this interpretation; the K_D observed against DADLE or normorphine was similar to that found when the inhibition of the contractile response of the guinea pig ileum was measured (10 μM); this is appropriate to a μ-receptor (26). By contrast, only 300 nM ICI 154129 is required to cause a 2-fold shift in the dose-response curve to DADLE in the mouse vas deferens, a tissue containing δ-receptors (26). Further evidence that the hyperpolarization of LC neurones is mediated exclusively by μ-type receptors comes from experiments with β-FNA. This is a selective μ-site-directed irreversible antagonist (27). Our finding that β-FNA was equally effective in blocking DADLE, DSLET, normorphine, EKC, and [Met^5]enkephalin indicates that all these agonists interact with μ-receptors.

Receptor occupancy and tissue response. Our estimates of naloxone K_D of about 2 nM are not dissimilar from those measured directly with tritiated ligands in homogenates (6–8, 25) or slices (28) of rat brain. This implies that the antagonist-binding site is resistant to homogenization and gross changes in its ionic environment. By contrast, our estimates of normorphine affinity (K_D about 10 μM) differ by 3 to 4 orders of magnitude from those based on ligand binding techniques (e.g., refs. 6–8, 25, and 28). Possible reasons for this discrepancy might be considered. For example, agonist affinities determined in binding assays are known to be markedly affected by the conditions. The concentration of normorphine required to displace one-half of bound [^{3}H]naloxone from rat brain homogenates is 15 nM in the absence of sodium ions, and 700 nM in the presence of sodium (6). In the same study, the naloxone EC_{50} was 1.5 nM in the presence or absence of sodium. Similarly, the addition of GTP reduces opiate agonist affinities in binding assays on homogenized tissues (e.g., refs. 29 and 30). On the other hand, there are differences between the concentrations of opioid agonists required to occupy half the binding sites and the concentration required to cause half-maximal effects even when both binding and effect are measured on intact cells under the same conditions (31, 32); such studies involved the δ-receptor of neuroblastoma × glioma hybrid NG 108-15 cells, and analogous information is not available for μ-receptors. The process of homogenization of rat brain eliminates cooperativity of ligand binding but does not change the affinity for agonists or antagonists (28).

General models which might account for the much lower affinity of receptors for agonists than antagonists have been extensively discussed; a popular one is a two-state model in which the binding site may or may not be coupled to an effector molecule (e.g., ref. 33). In such a scheme, agonists bind with a lower affinity than antagonists because part of the intrinsic free energy of their binding is applied conformationally to changing the effector. Such a model is compatible with the present results; however, as Thron (34) and Colquhoun (35) have pointed out, pharmacological techniques of the type used would probably not be able to distinguish between this and a simple one-state occupancy model.

In brief, the present experiments show that the action of opioids on the potassium conductance of single LC neurones can be interpreted in terms of an interaction with μ-type opioid receptors. The results indicate that agonist occupancy of only a small fraction of the receptors on one cell may be sufficient to inhibit its generation of action potentials.

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